MECHANISMS OF LUNG INFLAMMATION FOLLOWING EXPOSURE TO SWINE BARN AIR

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ABSTRACT

Occupational exposure to endotoxin-rich swine barn air induces respiratory diseases and loss of lung function. Barn exposure induces recruitment of pulmonary intravascular monocytes/macrophages (PIMMs) and subsequent increased host sensitivity to *Escherichia coli* LPS challenge. Therefore, to further clarify the biology of PIMMs we examined the role of recruited PIMMs in a rat *Escherichia coli*-induced lung inflammation model. Following sepsis, lung inflammation was induced with recruitment of PIMMs and subsequently, *Escherichia coli* LPS challenge exacerbated the lung inflammation with localization of multiple inflammatory cytokines in PIMMs to suggest their possible involvement in modulating lung inflammation in this model.

In order to delineate mechanisms of barn air induced lung dysfunction, a rat model of occupational exposure was characterized to show that one and five exposures to the barn environment induced acute lung inflammation and increased airway hyperresponsiveness (AHR). Following 20 exposures, AHR was dampened to indicate adaptive responses. Barn air contains high levels of endotoxin which led us to investigate its role in lung inflammation and AHR. Exposure of mice with either a functional TLR4 (WT) or non-functional TLR4 (mutants) to barn air revealed dependence of lung inflammation but not AHR on a functional TLR4.

I investigated whether exposure to barn air alters host responses to a subsequent microbial challenge. Following one day barn exposure and *Escherichia coli* LPS challenge, lung inflammation was exacerbated with increased granulocytes and IL-1β levels compared to one day barn exposed rats without *Escherichia coli* LPS challenge. However, increased granulocytes and IL-1β levels in barn exposed and *Escherichia coli* LPS challenged rats were not different from control rats treated with *Escherichia coli* LPS indicating a lack of priming effect of barn exposure. However, above results are suggestive of an underlying risk of increased lung inflammation following secondary microbial infection in naïve barn workers.

Lastly, I investigated the expression and activity of novel signalling molecules called *N*-myristoyltransferase and calcineurin in barn air and *E. coli* LPS induced lung inflammation models. Following one day barn exposure, increased protein expression but not activity of *N*-myristoyltransferase and calcineurin was shown. However, there is a need to identify the specific role of these two molecules in barn air induced lung inflammation. To conclude, animal models of barn exposure are useful tools to understand mechanisms of lung inflammation and AHR. However, there is still a need to examine endotoxin-independent nature of AHR and roles of other molecules of the innate immune system in regulating barn air induced effects.

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Dedicated to

My mother and father

For everything they have done for me

My brother and sisters

For their love and affection

My wife Shivaleela

For her tremendous support and encouragement

My son Dhruva

For his love and naughtiness that make me smile all the time

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LIST OF ABBREVIATIONS

AHR	Airway hyperresponsivness
ALI	Acute lung injury/inflammation
BALF	Broncho-alveolar lavage fluid
CAFO	Concentrated Animal Feeding Operations
CaN	Calcineurin
CFU	Colony forming unit
COPD	Chronic obstructive lung disease
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GC	Gadolinium chloride
HSC 70	Heat shock congnate protein 70
LAL	*Limulus* amoebocyte lysate
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
Mch	Methacholine
MRSA	Methicillin resistant *Staphylococcus aureus*
NF-κB	Nuclear factor kappa B
NIP 71	*N*-myristolytransferase inhibitor protein
NMT	*N*-myristolytransferase
PBS	Phosphate buffered saline
PIMMs	Pulmonary intravascular monocytes/macrophages
PIMP	Pulmonary intravascular mononuclear phagocytes
PIMs	Pulmonary intravascular macrophages
PKC	Protein kinase C
PM	Particulate matter

qRTPCR	Quantitative real-time reverse transcriptase-polymerase chain reaction
TLR	Toll-like receptor
TLV	Threshold limit value
TRAM	TRIF-related adaptor molecule
TRIF	Toll/IL-1 receptor-domain-containing adaptor inducing IFN-β

CHAPTER 1:REVIEW OF LITERATURE *

1.1. Introduction

Respiratory diseases of agricultural workers have been recognized as early as 1555. Although accidental death rates in agriculture are similar to mining and construction, occupational risks in farm work have received far less attention and investigation (reviewed in (Schenker *et al.*, 1998; Spurzem *et al.*, 2002)). In order to satisfy increased demand for food at a cheaper price, livestock farming has been industrialized. Although these modern, large-scale concentrated animal feeding operations (CAFOs) are efficient in food production at lower cost, they also pose a greater risk for the health of large number of workers with many impacts on the environment and communities living nearby. Emissions from CAFOs and application of waste manure onto the surrounding agricultural land could expose the nearby communities to many toxic gases, microbes, pesticides, veterinary antibiotics and chemicals. Often communities in the vicinity experience obnoxious odors, respiratory diseases, decreased health and property value, impaired mental health and reduced immune function (reviewed in (Radon *et al.*, 2007). Therefore, mechanisms of health effects of exposure to CAFO environments demand a thorough and systematic investigation.

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1.2. Changing face of the swine industry in Canada

Traditionally, swine farming was limited to small backyard activities involving mostly family members. However, in recent years it has transformed into large, industry-scale modern farming operations. Sharp decline in the numbers of farms and a higher demand for cheap food necessitated the raising of large number of pigs in small and confined buildings with modern animal farming approaches (Statistics Canada, 2001; Statistics Canada, 2007; Centner, 2003). These concentrated animal feeding operations (CAFOs) achieve the climate control, automation and specialized care for food animal rearing, resulting in lower costs of production. However, they pose potential occupational, environmental and community hazards and raise the need for public debates and legislative interference (reviewed in (Cole *et al.*, 2000; Mitloehner and Schenker, 2007; Radon *et al.*, 2007)).

Canada is among the top five pork exporters in the world with a total pork export of 970,000 tons in the year 2004, which translates into cash receipts of $4.2 billion for the year 2004, representing a 25% increase over the year 2003. However, hog production receipts amounted to $3.4 billion in the year 2006 and this decline is due to 12.7% lower price compared to the previous year (Statistics Canada-Agriculture Division, 2007). In the year 2005, Canada had 14.9 million hogs which is an increase of 1.7% over the previous year and pork export was expected to grow by 2% (US Department of Agriculture-Foreign Agricultural Service, 2006; Statistics Canada-Agricultural Division, 2006). Currently, the pork sector accounts for 30% of total livestock shipments and for 10% of all farm cash receipts in the Canadian farm economy. Further, swine farming had previously provided employment to 10,790 farm operators in Canada (Statistics Canada, 2001) and this number has recently declined to 9,245 for the year 2006 (Statistics Canada, 2007). Therefore, swine production is a major component of Canada's agricultural economy. Although the number of pigs has increased, the number of farms has shown a decline to indicate that fewer people are working longer shifts on the farms. Currently, small family-operated pig farms are making way for large scale facilities where thousands of pigs are raised in a single facility (Cole *et al.*, 2000). Large pig production operations require many full time workers who work 8 hour/day and 5 days/week and thus experience high intensity interrupted exposures to the barn air

(Wenger, 1999; Wenger *et al.*, 2005). However, still many workers may work only a few hours every day inside a pig barn.

1.3. Hazardous components of swine barn environment

The swine barn environment is very complex in composition (Donham *et al.*, 1986) and contains organic dust, plant materials (pollen grains, feed grains, hay and silage), animal origin materials (swine dander, hair, urine and pig proteins), microbial components (mite or their parts, bacteria, endotoxin, (1-3) β-D-glucan and fungal spores) and a number of gases such as ammonia, carbon dioxide, hydrogen sulphide and methane (Asmar *et al.*, 2001; Donham *et al.*, 1986; Donham and Popendorf, 1985). Therefore, although modern barns appear cleaner, the air inside these barns still carries toxic molecules which are harmful to the workers (Cormier *et al.*, 2000). These modern CAFOs generate a large amount of waste materials on-site (288 million tons in the United States annually) that produces annoying odor due to many gases, organic, inorganic and microbial components. A large number of these hazardous components do not have occupational exposure limits. However, The Occupational Safety and Health Administration , The American Conference of Government Industrial Hygienists and The National Institute for Occupational Safety and Health have provided few guidelines to monitor their levels (Cole *et al.*, 2000; Institute for Agriculture and Trade Policy, 2007; US Environmental Protection Agency, 2007; American Public Health Association, 2008).

1.3.1. Vapors and gases in swine barn environment

Although storage and handling of animal waste generates as many as 150 potentially toxic gases, exposure to ammonia, hydrogen sulfide and carbon dioxide are considered important for their health effects on barn workers (Schenker *et al.*, 1998; Heederik *et al.*, 2007). Most often, the concentration of these gases is below the level of occupational exposure limits recommended by the regulatory authorities (Von Essen and Donham, 1999; Cole *et al.*, 2000). These gases could support the growth of microorganisms found in the swine barn as well as affect the buildings and farm equipments causing corrosion (Swine Odor Task Force, 2008).

Ammonia is produced from the breakdown of urea from pig urine and its concentration most commonly exceeds the Threshold Limit Values (TLV) (Donham and Popendorf, 1985). The TLV for ammonia is 25-50 ppm. Ammonia is water-soluble and is usually absorbed in the upper respiratory tract and under high humidity, it can also adsorb onto aerosols to travel deep into lungs. Ammonia is an irritant to the eyes, skin, mucous membrane and upper respiratory tract (reviewed in (Cole *et al.*, 2000)) (Donham and Popendorf, 1985).

Hydrogen sulfide is produced from anaerobic degradation of the liquid manure and is an irritant to the eye and respiratory tract. Acute exposure to high-levels of hydrogen sulfide is life threatening to humans and animals while chronic low-grade exposures could result in increased susceptibility to respiratory infections and also cause photophobia, anorexia, and nervousness. However, proper ventilation and careful handling of the manure could limit its levels to below 20 ppm to provide a safer work environment (Swine Odor Task Force, 2008).

Increased levels of carbon monoxide could be a result of improperly vented or malfunctioning space heaters. Higher carbon monoxide levels (2,000 ppm for chicken and 4,000 ppm for pigs) are fatal while lower levels (200-300 ppm) could reduce the growth of farm animals by 25% (Swine Odor Task Force, 2008). Carbon dioxide levels in swine barns are between 1,400-5,000 ppm under normal conditions while build-up of extremely higher levels such as 100,000 ppm result in dizziness, anxiety, staggering, and unconsciousness in pigs (Swine Odor Task Force, 2008).

Methane is usually found in the range of 3-35 ppm and the recommended safety limit is 25 ppm. However, recent research has shown that maintaining methane levels at or below 10 ppm may more readily prevent the health risks to humans and pigs. The lethal concentration of methane is around 3,000 ppm and methane is the most common noxious gas in the swine barn that causes irritation and tissue damage to animals and humans (Schenker *et al.*, 1998; Swine Odor Task Force, 2008)

1.3.2. Particulate matter

Particulate matter generated from CAFOs includes feed material, fecal matter, skin cells and products of microbial degradation of feed and fecal matter. Feed material

includes plant proteins, starches, carbohydrates, minerals, amino acids and veterinary antibiotics. The total suspended particulates could be divided into two categories. First fraction comprised of dust particles smaller than 100 µm is called, "inhalable dust fraction" and the second fraction made up of dust particles smaller than 3.5 µm is called the "respirable dust fraction". The two other important fractions of particulate matter (PM) are called, PM_{10} and $PM_{2.5}$. PM_{10} is the dust fraction with a particle size of less than 10 µm in diameter and $PM_{2.5}$ is less than 2.5 µm in diameter. Currently, particles in the size range of less than 0.1µm are receiving attention because of their deposition deep into the lung (Iowa State University and The University of Iowa Study Group, 2007).

1.3.3. Microbial components in the swine barn environment

Microbial components of the swine barn are mainly present in two different fractions. The first one is airborne and settled particulate matter of biological origin and is called, "organic dust". The second portion consisting of particles of biological origin held suspended in the air are called, "bioaerosols". Both organic dust and bioaerosols are heterogeneous in composition and mainly contain microbial components. Microbial components include bacteria, fungi, spores of bacteria and fungi, viruses, mammalian cell debris, microbial products, pollens, dust mites, pig urine proteins and aeroallergens. These microbial components vary in size from as big as 30-50 µm (pollens) to as small as 0.001-0.05 µm (viruses) (Iowa State University and The University of Iowa Study Group, 2007).

Among the bacteria present in the barn, Gram-positive bacteria predominate (68-96%) over Gram-negative bacteria (7-53%) and from among the total number of bacteria, non-culturable ones predominate over culturable ones (reviewed in (Cole *et al.*, 2000)). Some of the bacterial and mold genera found in the swine barn environment are *Pseudomonas*, *Enterobacter, Flavobacterium, Bacillus, Corynebacterium, Aspergillus, Scopulariopsis, Penicillium, Geotrichum, Mucor* and *Fusarium*. Some of the yeasts found in swine barn environments include *Candida, Cryptococcus, Toruopsis, Trichosporon, Rhodotorula,* and *Hansenula*. Bacteria and fungi found in the barn environment could be either infectious or non-infectious species and even the non-infectious species have been shown to cause respiratory illness (Schenker *et al.*, 1998; Iowa State University and The

University of Iowa Study Group, 2007). The bacterial components in the bioaerosol include endotoxins, exotoxins, peptidoglycans, lipoteichoic acids and bacterial DNA bearing CpG motifs. Apart from these, fungal products such as conidia and microconidia, hyphal fragments, mycotoxins and glucans are also present in the barn. Pigs raised in such a barn environment act as an important host and possibly transmit many zoonotic diseases such as Hepatitits E virus, Nipah virus and Influenza virus to humans (Cole *et al.*, 2000; Balayan *et al.*, 1990; Meng *et al.*, 1997; Myers *et al.*, 2007; Bellini *et al.*, 2005).

Endotoxin is a lipopolysaccharide (LPS) component of the outer cell wall of Gram-negative bacteria and is ubiquitously present (Schenker *et al.*, 1998; Radon, 2006). LPS molecule has a biologically active part (lipid A) and a hydrophilic polysaccharide moiety. Endotoxin or LPS is a potent pro-inflammatory molecule and upon exposure could produce systemic as well as local effects (Abbas and Lichtman, 2005b; Schwartz, 2001; Singh and Schwartz, 2005). CAFOs are known to have some of the highest concentrations of endotoxin resulting in occupational exposure of workers. The environmental samples of endotoxin are collected using either liquid impingers or air sampling filters (Duchaine *et al.*, 2001) and concentrations are analyzed using the *Limulus* amebocyte lysate (LAL) bioassay (Thorne *et al.*, 1997; Douwes *et al.*, 1995). The mean endotoxin exposure in swine CAFOs is highly variable and it is difficult to interpret endotoxin exposure since there are no occupational exposure limits. However, various studies have established no-effect levels for endotoxins in the range of 1-20 ng/m^3 to 170-180 ng/m^3 (reviewed in (Heederik *et al.*, 2007; Cole *et al.*, 2000)). Although most components of the barn air are detrimental, endotoxin is believed to be central to the health effects on exposed individuals (Jagielo *et al.*, 1996b; Schwartz, 2001).

1.4. Effects of exposure to swine barn environment

The effects of exposure to complex swine barn environments are many. Apart from the negative impacts on the health of exposed individuals, swine CAFOs or CAFOs in general have a negative impact on the social and economic well being of the rural communities (Osterberg and Wallinga, 2004). Epidemiological studies have linked symptoms of impaired mental health to living in the vicinity of CAFOs (Schiffman *et al.*, 1995). Further, CAFOs have shown to inflict many social impacts on the communities that live near them. The bad odor emanating from the swine barns could limit the joys of rural outdoor activities, reduce the social gatherings, reduce the price of the land, buildings and other property values and also create conflicts between those who own and or operate CAFOs and those who do not (reviewed in (Donham *et al.*, 2007)). These CAFOs are also known to be located disproportionately in areas populated by people of color, nonwhite and low income communities who experience discrimination and may also experience higher susceptibility due to their poor-housing conditions and health, lack of access to healthcare and low income (Wing *et al.*, 2000; Mirabelli *et al.*, 2006).

1.4.1. Respiratory diseases of swine farmers

Respiratory diseases in agricultural farmers are one of the earliest recognized occupational hazards (Schenker *et al.*, 1998). Among the agriculture workers, swine farmers report higher prevalence of occupational respiratory symptoms (Iversen *et al.*, 1988). Exposure to hazardous toxic molecules in the barn environment is considered a risk factor for the development of chronic respiratory symptoms and lung dysfunction (Zejda *et al.*, 1993). Exposed workers report significantly higher frequencies of respiratory symptoms, chest illness, cold and pneumonia (Asmar *et al.*, 2001; Zejda *et al.*, 1994). The severity of respiratory symptoms in swine barn workers increases during the winter due to the reduced ventilation and is also related to the number of working hours (Iversen *et al.*, 2000). Multiple regression analysis of 16 different environmental parameters showed that endotoxin is related to FEV1 in a dose-dependent manner with increasing exposures causing more decrease in FEV1 (Donham *et al.*, 1989). Swine farmers who have worked in the barn for many years suffer from various respiratory

diseases (Table 1.1; compiled from (Donham, 2000; Schenker *et al.*, 1998; Von Essen and Romberger, 2003). The smoking could further intensify the signs respiratory diseases (Donham *et al.*, 1984a) and female workers appear to have a higher risk for the effects of swine barn exposure compared to males (Senthilselvan *et al.*, 2007).

1.4.2. Exposure to swine barn environment and lung dysfunction

Swine farmers experience annual decline in lung function (Senthilselvan *et al.*, 1997a) that is associated with endotoxin exposure (Vogelzang *et al.*, 1998). Previous studies have recorded reductions in expired flow rates in barn workers (Donham *et al.*, 1984b; Zejda *et al.*, 1993; Bongers *et al.*, 1987; Haglind and Rylander, 1987). Furthermore, barn workers also exhibit increased AHR and airway inflammation (Zhou *et al.*, 1991; Larsson *et al.*, 1994). The longitudinal decline in lung function in swine barn workers has been linked to air contaminants (Schwartz *et al.*, 1995) and a dose-response relationship exists between decline in lung function and endotoxin and ammonia levels in the barn air (Donham *et al.*, 1989; Dosman *et al.*, 1988). Exposure to the barn organic dust causes airway inflammation and increased airway resistance both in humans and animal models apart from contributing to the exacerbation of asthma (Kennedy *et al.*, 1987; Lorenz *et al.*, 2001; Michel *et al.*, 1989; Jagielo *et al.*, 1998; Michel *et al.*, 1996). These observations show that the barn air contains toxic molecules which induce lung dysfunction in pig barn workers. However, cell and molecular mechanisms of lung inflammation and lung dysfunction remain to be discerned.

Table 1.1. Respiratory diseases of swine farmers (compiled from (Donham, 2000; Schenker *et al.*, 1998; Von Essen and Romberger, 2003)).

Upper airway diseases
Rhinitis (allergic/irritant)
Mucous membrane irritation of the throat
Pharyngitis
Sinusitis
Interstitial lung disease
Organic dust toxic syndrome
Hypersensitive pneumonitis
Alveolitis
Pulmonary edema
Lower respiratory tract disease
Organic dust toxic syndrome
Bronchitis (acute/subacute/chronic)
Asthma-like syndrome
Occupational asthma
Chronic obstructive pulmonary disease (COPD)

1.4.3. Effect of CAFOs on the environment, communities and farm animals

CAFOs in general have diverse negative impacts on the environment mainly due to the production of a huge amount of animal waste. Manure and dead pigs produced from swine CAFOs contain a large amount of antimicrobials, microbes, nutrients, hormone residues and heavy metals and produce bad odor (reviewed in (Cole *et al.*, 2000; Iowa State University and The University of Iowa Study Group, 2007). Because it is common to use antimicrobials in CAFOs, there is a threat of evolution of antibiotic resistant bacteria. Observation of a higher prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in pigs and transmission of MRSA strains between pigs and humans support the threat of evolution of antibiotic resistant bacteria from CAFOs (Huijsdens *et al.*, 2006; de Neeling *et al.*, 2007). Further, the application of liquid manure to the agricultural land and accidental leakage of manure storage lagoons pose the risk of spread of antibiotic resistant bacteria to the nearby communities, ground water, soil and air. Taken together, waste materials generated from swine CAFOs pose a serious impact on the surrounding environment as well as health of the community in the vicinity (Cole *et al.*, 2000; Osterberg and Wallinga, 2004).

Since CAFO environment affects the health of the exposed individuals, it is interesting to study if there are any similar effects on the health and productivity of exposed farm animals. The barn environmental pollutants are known to affect porcine and bovine immune systems (Raszyk *et al.*, 1997). Interestingly, a significant association between lung diseases of pigs and lung function of swine farmers has been recorded (Bongers *et al.*, 1987).

Various hazardous components of the swine barn environment have an effect on the growth, productivity and health of the pigs being raised on CAFOs. For example, ammonia is the most noxious gas produced in the CAFO environment (Swine Odor Task Force, 2008) and at concentrations below 100 ppm, it irritates respiratory mucosa, eyes and affects the growth of young animals (Lillie, 1972; Curtis *et al.*, 1975). Exposure to ammonia also causes lesions in tracheal and nasal epithelium (Drummond *et al.*, 1978b), reduced clearance of bacteria from lungs, affects mucociliary transport, alveolar macrophage function and also results in reductions in weight gain (Drummond *et al.*,

1978a; Drummond *et al.*, 1981a; Drummond *et al.*, 1981b; Lillie and Thomson, 1972). These are respiratory effects of ammonia which is present in the pig barns. Therefore, it is relevant to the animal effects of pig barn air.

Hydrogen sulfide in the barn arises due to anaerobic decomposition of protein and other organic material. The usual concentrations in the barns (< 10 ppm) are harmless while release of hydrogen sulfide from manure agitation could reach concentrations as high as 1000 ppm or higher (Lillie, 1972; Donham, 2000). Acute exposure of pigs to hydrogen sulfide gas produce a range of clinical effects ranging from no significant changes (50-100 ppm), distress (250 ppm), semi comatose (500-700 ppm), intermittent spasms, convulsions and even death at concentrations ≥1000 ppm (Curtis *et al.*, 1975; O'Donoghue, 1961).

The barn environment contains significant levels of organic dust to which both workers and animals get exposed (Donham, 2000). Airborne dust has been linked to decreased growth rate, increased respiratory diseases and lung damage in pigs (International Commission of Agricultural Engineering, 1994) while both airborne bacteria and dust have been linked to atrophic rhinitis (Robertson *et al.*, 2007). Experimental inhalation of feed flour dust has been shown to induce bronchial airway inflammation in pigs (Urbain *et al.*, 1999). Barn dust is strongly linked to reduced growth and respiratory health of pigs while reduction in airborne pollutants improves efficiency of pigs (reviewed in (Pedersen *et al.*, 2000)). Because pigs have been raised on CAFO environment for many years and also exposed to the self-antigens (pig proteins), there are possibilities of tolerance to the effects of CAFO environment.

1.5. Experimental exposures to swine barn air

1.5.1. In vitro studies on the effects of swine barn dust

Several researchers have shown the inflammatory potential of swine barn dust or air in many *in vitro* experiments (Palmberg *et al.*, 1998; Wang *et al.*, 1999; Romberger *et al.*, 2002). These *in vitro* studies are a valuable tool to understand the effects of swine barn air or dust on a variety of lung cells. These studies provide an opportunity to control many variables to facilitate identification of cellular and molecular pathways that regulate lung innate response to swine barn air. Swine dust induces release of IL-8 in normal

human bronchial epithelial cells, human pulmonary epithelial carcinoma cell line (A549) and in human alveolar macrophages (Palmberg *et al.*, 1998). Swine dust is almost as potent as lipopolysaccharides in stimulating cytokine release from alveolar macrophages *in vitro* (Wang *et al.*, 1999). Recent data showed that swine barn dust activates protein kinase C (PKC) to induce secretion of IL-8 and IL-6 from airway epithelial cells and promotes adhesion of lymphocytes through up regulation of ICAM-1 (Romberger *et al.*, 2002; Mathisen *et al.*, 2004). Swine barn dust can also directly activate T-lymphocytes (Muller-Suur *et al.*, 2002). Hog barn dust extract increases the baseline ciliary beat frequency of airway epithelial cells in a concentration and time-dependent and endotoxin-independent manner (Wyatt *et al.*, 2008). Further, hog barn dust extract has been shown to slow down the migration of airway epithelial cells through a PKCα-dependent mechanism (Slager *et al.*, 2007). A single exposure of promonocytic THP-1 cells to organic dust induced TNF-α, IL-6, CXCL8 (IL-8) and IL-10 secretion and TNF-α secretion was independent of endotoxin. Pretreatment of peripheral blood monocytes with dust extract, LPS and peptidoglycan could reduce the secretion of TNF-α while IL-6 secretion was only reduced in those cells pretreated with dust extract and LPS. Interestingly, following pretreatment and restimulation, CXCL8 and IL-10 levels remained persistently elevated. PKC isoenzymes (α, ε, δ and ζ) were elevated following a single treatment with the swine confinement organic dust, while with repeat exposure their levels were attenuated indicating their possible involvement in adaptation mechanism. Organic dust induced TNF-α secretion was significantly reduced with PKCα and PKCε inhibition (Poole *et al.*, 2007). Although *in vitro* studies provide important molecular data, we still need *in vivo* studies to understand cellular and molecular responses in intact organisms exposed to pig barn air.

1.5.2. Effects of acute single exposure to the swine barn air: human studies

To better understand the negative effects of exposure to swine barn air, many researchers have exposed healthy volunteers to the swine barn air for a short period of time (2-5 hours, once). This study model mimics the lung response of naive workers following first exposure to the swine barn air. Single two to five hours of exposure of naïve, healthy volunteers to swine barn air is shown to induce bronchial responsiveness

(Malmberg and Larsson, 1993), fever, malaise and drowsiness (Larsson *et al.*, 1994). Across the shift change in lung function during exposure has shown to be an important predictor of longitudinal changes in lung function in swine confinement workers (Kirychuk *et al.*, 1998). Furthermore, a 75-fold increase in neutrophils, a two-three fold increase in mononuclear cells and a significant increase in eosinophils, fibronectin and albumin levels in bronchoalveolar lavage fluid (BALF) (Larsson *et al.*, 1994) and levels of IL-1β, IL-1 receptor antagonist , IL-6 and TNF-α increased in the serum of the exposed naïve volunteers. The changes in IL-1 receptor antagonist levels correlated with changes in FEV1, bronchial responsiveness, oral temperature and white blood cell count while IL-1β levels correlated with oral temperature (Wang *et al.*, 1998). Further, a single exposure to barn air also caused thickening of nasal mucosa, increased numbers of neutrophils in nasal lavage and BALF, increased numbers of macrophages, lymphocytes, eosinophils and the levels of IL-8 in the BALF (Larsson *et al.*, 1997; Cormier *et al.*, 2000). Interestingly, levels of IL-8, a potent chemoattractant for neutrophils, correlated with increase in neutrophils in the nasal lavage fluid (Larsson *et al.*, 1997). It is obvious that single exposure to the barn air can activate an inflammatory response in human lungs.

Although the major health effects of working in swine confinement facilities result from the inhalation of the toxic components of the barn air, swine workers are also at risk for various other diseases, injuries and dangers associated with the general farm work environment. Swine workers are at risk for infectious diseases, noise induced hearing impairment and subsequent reduced safety, thermal stress (heat/cold), electrocution, fires and explosions, animal bites, needle sticks, chronic pain, emotional stress and fatigue (Iowa State University and The University of Iowa Study Group, 2007).

1.5.3. Animal exposure studies

Although data from studies involving human volunteers has shown induction of lung inflammation following exposure to the barn air, animal studies are critically needed to better understand cellular and molecular changes. So far, there have been very few animal model studies to elucidate the mechanisms of barn air-induced lung dysfunction.

For example, rabbits and guinea pigs maintained for 12 months in a confined nursery-grower unit showed diffuse interstitial histiocytic pneumonia, epithelial hyperplasia and metaplasia of tracheal and nasal turbinates, with sub mucosal infiltration of plasma cells and heterophils (Donham and Leininger, 1984). Interestingly, blood from these test animals contained serum precipitins to dust extract from the swine confinement building (Donham and Leininger, 1984). Rubinstein and Von Essen showed that hog barn dust extract increases macromolecular efflux from the *in situ* hamster cheek pouch and corticosteroids could attenuate the hog barn dust extract induced macromolecular efflux. The plasma exudation in the form of macromolecular efflux is believed to play an important role in upper airway dysfunction induced by exposure to swine barn dust (Rubinstein and von Essen, 2006). This macromolecular efflux was also shown to be in part due to reactive oxygen species that were inactivated by the enzyme catalase (Rubinstein and von Essen, 2006). Therefore, animal exposure studies particularly those that mimic the occupational exposure pattern of swine barn workers are of great value. These models help us in predicting the exposure-effects and provide insights into *in situ* cell and molecular mechanisms of lung dysfunction induced following exposure to swine barn air.

1.6. Fundamentals of innate immunity in lung inflammation

1.6.1. Innate immunity in the lung

The lung is in continuous contact with the external environment through inhaled air (Martin and Frevert, 2005). Surprisingly, such a vulnerable organ is protected from microbial infections by highly efficient innate immune system that acts as the body's first line of defense. Innate immune system employs pattern recognition receptors that specifically recognize certain molecular patterns on microbes, termed microbial associated molecular patterns. Recognition of microbes is followed by activation of the innate immune system that leads to removal of microbes or their products and further stimulates the adaptive immune system. The effector arm of the innate immune system consists of physical barriers to the entry of microbes (epithelial layer, defensins and intra-epithelial lymphocytes), circulating cells (neutrophils, macrophages and NK cells), proteins (complement, collectins, C-reactive proteins and coagulative proteins) and

cytokines secreted mostly by activated immune cells such as macrophages and dendritic cells (Abbas and Lichtman, 2005b)

1.6.2. Physical defenses and anti-microbial compounds in the lung

The nose and upper respiratory tract act as a filter to remove most of the inhaled particles before they pass down to the lower respiratory tract (Rastogi *et al.*, 2001). Further, the pattern of air flow due to the anatomic nature of turbinates, closure of glottis, cough and sneeze reflex, ciliary beat, tight-junctions between airway epithelial cells and mucociliary clearance help to protect the respiratory system against microbial infections (Zaas and Schwartz, 2005; Chilvers and O'Callaghan, 2000; Holt, 2000; Randell and Boucher, 2006; Rastogi *et al.*, 2001). Following entry of an infectious organism, airway epithelium, submucosal glands and phagocytic immune cells secrete anti-microbial peptides (reviewed in (Cohn and Reinero, 2007); (Bals and Hiemstra, 2004; Rastogi *et al.*, 2001) that are capable of either inhibiting or killing bacteria, fungi and enveloped viruses (Koczulla and Bals, 2003). Anti-microbial peptides are also involved in linking innate and adaptive immunity, regulation of inflammation, wound healing and angiogenesis (reviewed in (Bals and Hiemstra, 2004).

1.6.3. Recognition of microbes and microbial products by innate immune system

Microbes that evade physical barriers and the action of anti-microbial compounds are sensed by pattern recognition receptors as danger signals. Airway epithelium, alveolar macrophages, dendritic cells and other immune cells employ pattern recognition receptors to specifically identify unique pathogen associated molecular patterns on each class of microbes or their products (Akira *et al.*, 2006; Zaas and Schwartz, 2005). The major class of pattern recognition receptors is known as Toll-like receptors (TLRs) which are present on both epithelial cells and leukocytes (Zaas and Schwartz, 2005; Bals and Hiemstra, 2004).

The founding member of TLR family, Toll was first discovered in fruit fly, *Drosophila melanogaster* (Anderson *et al.*, 1985) and TLRs are evolutionarily conserved. Homologous receptors are found in plants, insects, worms such as *Caenorhabditis elegans* and vertebrates. To date, a total of 13 mammalian homologues of Toll have been

identified of which TLR4 is the principle receptor for endotoxin recognition and is also implicated in recognizing fungal and parasite components, envelop proteins from viruses and host components such as heat-shock protein 60 and 70 and fibrinogen. The other members of the TLR family are involved in recognizing a wide variety of ligands (reviewed in (Akira *et al.*, 2006; Albiger *et al.*, 2007)).

1.6.4. TLR4 expression in the lung

TLR4, the most widely researched member of the TLR-family is central to the host response to LPS or endotoxin (Bals and Hiemstra, 2004). TLR4 expression in normal and inflamed lungs has been studied using immunohistochemistry at both light and electron microscopy levels. TLR4 is expressed in lung monocytes and macrophages, neutrophils, eosinophils, alveolar septa, bronchiolar epithelium, endothelium of large and peribronchiolar blood vessels as well as in Type I and II alveolar epithelial cells, microvascular and macrovascular endothelium and pulmonary intravascular macrophages. Interestingly, TLR4 has been localized in cytoplasm and nucleus alone as well as in conjunction with LPS of lung cells including the endothelial cells (Janardhan *et al.*, 2005; Wassef *et al.*, 2004; Singh *et al.*, 2006).

1.6.5. Endotoxin induced lung inflammation; an overview

1.6.5.1. Host recognition of endotoxin through TLR4-mediated signaling

Endotoxin or LPS- is one of the potent pro inflammatory molecules and is ubiquitously present in commercial milk samples, domestic water, house dust as well as farming and industrial work environments (reviewed in (Michel, 2000))(Schwartz *et al.*, 1995; Christiani *et al.*, 1994; Glindmeyer *et al.*, 1994; Laitinen *et al.*, 2001; Abbas and Lichtman, 2005b). Endotoxin-induced lung inflammation or airway disease is the most extensively studied model of lung injury (Hauswirth and Sundy, 2004). The presence of endotoxin is sensed by TLR4 which initiates a cascade of signaling events (Figure 1.1). Before being recognized by TLR4, LPS needs to be bound to lipopolysaccharide binding protein (LBP) and then with the help of CD14 (membrane bound and soluble) and MD-2, TLR4 signaling is initiated. Subsequently, adapter proteins MyD88, TIR domain-containing adapter protein (TIRAP) as well as IL-1 receptor-associated kinase (IRAK) are recruited into the signaling complex. Following autophosphorylation, IRAK dissociates itself from MyD88 to activate TNF-R-associated factor-6 (TRAF6). Then TRAF-6 detaches IκB to assist in the nuclear translocation of NF-κB and subsequent production of inflammatory cytokines such as TNF-α, IL-1β and IL–6. TLR4 signaling could also occur in MyD88-independent manner (Bals and Hiemstra, 2004; Abbas and Lichtman, 2005b; Takeda and Akira, 2004).

1.6.5.2. Endotoxin-induced clinical signs and lung function changes

Inhalation of pure endotoxin or endotoxin-rich organic dust induces fever, chills, headache, fatigue and malaise as well as chest tightness, cough, dyspnea, joint and muscle pain (reviewed in (Thorn, 2001; Michel, 2000)). Further inhaled endotoxin increases airway hyperresponsiveness (AHR) to nonspecific stimuli, decrease in FEV1 and increased bronchoconstriction upon histamine challenge (reviewed in (Reed and Milton, 2001; Michel, 2000)). Many of the symptoms of endotoxin-induced airway disease are similar to an acute asthma event and inhalation of endotoxin

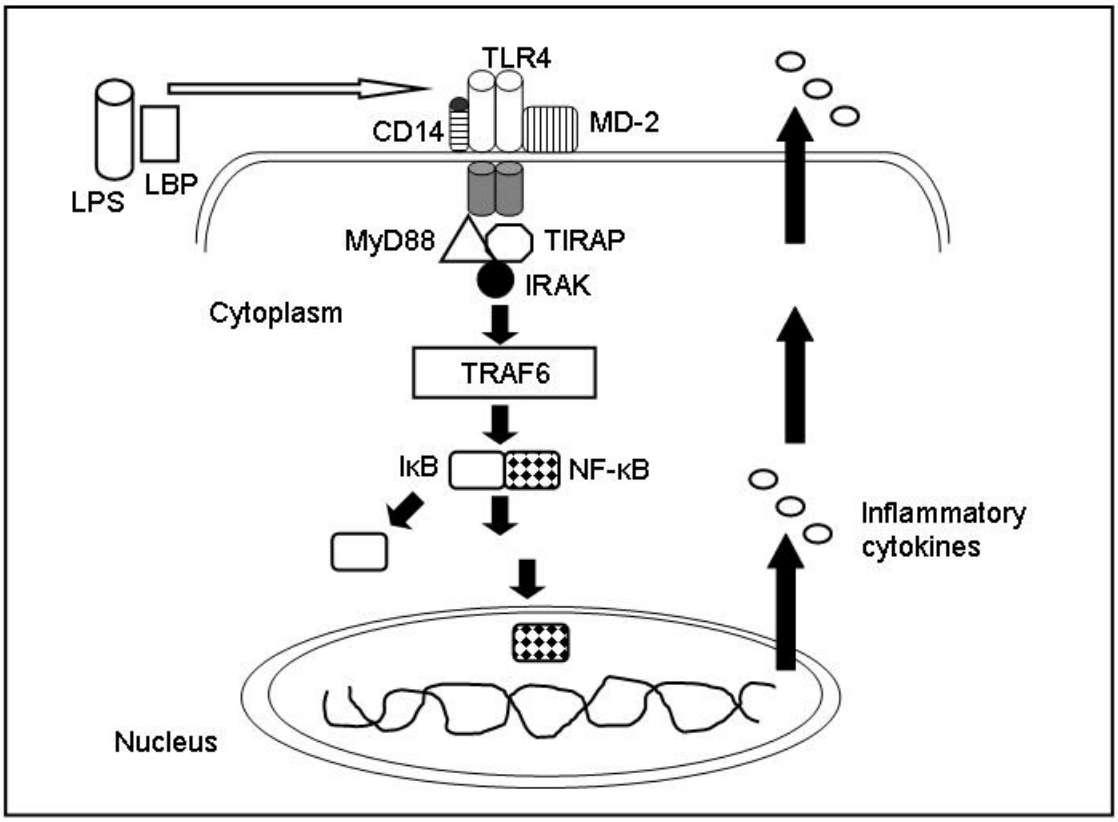

Figure 1.1. An overview of host-recognition of endotoxin or LPS through TLR-4 mediated signaling.

is known to cause and exacerbate asthma and other airway diseases (Schwartz, 2001). The endotoxin concentration in swine, poultry and other occupational work settings is linked to lung function changes (Schwartz, 2001; Haglind and Rylander, 1984; Donham *et al.*, 1989; Rylander *et al.*, 1985; Kennedy *et al.*, 1987; Thelin *et al.*, 1984). Interestingly, the competitive antagonism of TLR4-signaling in a chronic LPS-induced airway disease model has shown to prevent the development of AHR and airway remodeling (Savov *et al.*, 2005). However, endotoxin-induced lung functional changes display large variation between subjects (Kline *et al.*, 1999) and also the requirement for TLR4-mediated signaling for lung inflammation and lung function changes depends on the nature of the environmental toxin (Hollingsworth *et al.*, 2004). Further genes other than TLR4 have been proposed to be important in endotoxin-induced lung inflammation and airway changes (Lorenz *et al.*, 2001). These observations underscore the importance of expanding our understanding of mechanisms of endotoxin and swine barn air-induced lung functional changes.

1.6.5.3. Cell and molecular changes in endotoxin-induced lung inflammation

Endotoxin primarily activates alveolar macrophages and type II epithelial cells in the lung leading to the production of early response inflammatory cytokines (TNF-α and IL-1β), chemokines (IL-8) and up regulation of various adhesion molecules to result in the recruitment of neutrophils and monocytes as well as increased vascular permeability and edema formation (Maus *et al.*, 2002; Thorn, 2001). The activated neutrophils and inflammatory cytokines together employ various enzymes and reactive oxygen/nitrogen species to orchestrate killing and or elimination of microbial etiology. These complex sets of events not only eliminate the danger signals but also initiate the resolution and healing process through elaboration of anti inflammatory cytokines such as IL-10 and TGF-β and growth factors (reviewed in (Thacker, 2006)). Therefore understanding the cell and molecular mechanisms of endotoxin induced lung inflammation and lung dysfunction is critical and serves as the initial step in delineating the molecular mechanisms of swine barn air induced lung inflammation.

1.6.5.4. Genetics of innate immune responses to endotoxin

Endotoxin-induced airway and inflammatory responses have shown inter-individual variations (reviewed in (Michel, 2000))(Arbour *et al.*, 2000). Historically the C3H/HeJ strain of mice was identified with innate resistance to endotoxin effects (Sultzer, 1968) and later a missence mutation in *Tlr4* gene was attributed to the same (Poltorak *et al.*, 1998). Subsequently, TLR4-polymorphisms in 10% of the human population were shown to have attenuated *in vitro* inflammatory and *in vivo* airway responses to LPS (Arbour *et al.*, 2000). Recently, importance of TLR4 in endotoxin-induced lung inflammation has been documented (Hollingsworth *et al.*, 2004; Savov *et al.*, 2005). Further how polymorphisms in different TLRs could affect many human diseases and differences between human and mice TLRs has been reviewed (Schwartz and Cook, 2005; Chaudhuri *et al.*, 2007). Although endotoxin has been strongly linked to grain dust and swine barn air induced health effects (George *et al.*, 2001; Vogelzang *et al.*, 1998), genetic control of lung inflammation and lung function changes following exposure to the complex swine barn air are not clear.

1.6.6 Inflammatory disease conditions of lung

1.6.6.1. Acute lung inflammation

Acute lung inflammation (ALI) is an inflammatory condition of the lung that arises due to many causes including Gram-negetaive bacterial infections to result in high morbidity and mortality (Matthay *et al.*, 2003). ALI is characterized by increased expression of pro-inflammatory cytokines and chemokines, infiltration of inflammatory cells such as neutrphils and monocytes, damage to the lung endothelium and epithelium and development of interstitial edema (Abraham *et al.*, 2000; Ware and Matthay, 2000). ALI is a common lung inflammatory condition following exposure to microbes or other irritants, still lacks effective treatments and hence understanding its pathophysiology is important.

1.6.6.2. Chronic lung diseases

Asthma, chronic bronchitis, emphysema and many occupational lung diseases such as silicosis, byssinosis, berylliosis and asbestosis are categorized as chronic lung diseases with chronic lung inflammation as the underlying feature. Chronic lung inflammation is characterized by persistent inflammation due to one or many causes and chronic obrastructive pulmonary disease is a typical example of the same. Prolonged inflammation in the lung is usually seen in long-term tobacco smoke exposure, occupational exposure to work place pollutants (Ameille *et al.*, 2006), bacterial and viral infections and allergic lung disases (reviewed in (Thacker, 2006)). Chief cellular infiltrates in chronic lung inflammation include lymphocytes, macrophages and plasma cells. Persistently elevated pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and release of collagenase and elastase from activated macrophages result in much of the pathology including damage to the connective tissue. Depending on the etiology and many other factors, chronic lung inflammation results in granulation tissue formation, fibrosis, hypertrophy of airway smooth muscle and bronchoconstriction. Most of these tissue changes may result in airflow limitation as well as annual decline in lung function (Thacker, 2006; Rushton, 2007).

1.6.6.3. Sepsis-induced lung inflammation

Sepsis is defined as a clinical syndrome characterized by both infection and systemic inflammatory response due to live bacteria or their products (Villar *et al.*, 2004; Vincent, 2008). Sepsis induces damage to many organs including lung.
Lung is prone to many infectious and inflammatory conditions due to its involvement in respiration. Sepsis-induced lung inflammation is characterized by excessive lung tissue recruitment of neutrophils (Guo *et al.*, 2002), complement activation (Huber-Lang *et al.*, 2002), activation of pro-coagulant pathways, increased productuoin of pro-inflammatory cytokines (Cohen, 2002) and development of interstitial edema (Martin and Bernard,

2001). However, exact mechanisms of sepsis-induced lung inflammation as well as sepsis-induced multi-ogran failure are unclear.

1.6.6.4 Asthma

Asthma is a complex disease affecting the conducting airways with multiple etiologies. Asthmatic airways excessively contract in response to external or endogenous stimuli (Holgate, 2008; Kannan and Deshpande, 2003). Further, asthma presents allergic airway inflammation with eosinophils, neutrophils, CD4+ T-lymphocytes and mast cells as the main cellular infiltrates. Asthma develops after allergic sensitization to one or many environmental allergens and subsequent Th2 cytokine production. However, in chronic and more severe forms, Th1 response (TNF-α and IFN-γ secretion) and abnormal airway epithelium with airway remodeling are also seen (Holgate, 2008). Many occupational work exposures including agricultural farm work are considered a risk factor for the development and exacerbation of asthma (Di *et al.*, 2007; Singh and Schwartz, 2005). Therefore, asthma is an important inflammatory disease condition of the lung.

1.7. Lung macrophages and their role in lung inflammation

Macrophages and epithelial cells are the principle targets of endotoxin regardless of the route of entry of endotoxin (Thorn, 2001; Reed and Milton, 2001). Macrophages are present in abundance in the lung and are mainly involved in sensing endotoxin through TLR4. In general, macrophages can ingest bacteria, protozoa, fungi, helminthes, tumor and virus-infected cells and normal cells undergoing apoptosis to bring about microbial elimination and antigen presentation. They are also involved in wound healing, metabolism of cholesterol and regulation of hematopoiesis (Sasmono and Hume, 2004). Once activated, they secrete cytokines and chemokines. These mediators are engaged in initiation and resolution of inflammation as well as interaction with the adaptive immune system (Gordon and Read, 2002; Aderem and Ulevitch, 2000; Medzhitov and Janeway, Jr., 2000).

Lung macrophages are classified based on their anatomical locations into three populations; alveolar, interstitial and intravascular macrophages. Alveolar macrophages

provide the first line of defense against inhaled microbes (Hauschildt and Kleine, 1995). Interstitial macrophages are present within the lung stroma and are smaller with lesser phagocytic abilities. Pulmonary intravascular macrophages (PIMs) are mature mononuclear phagocytes found strongly adhering to the endothelium of the lung microvessels in certain species namely, equines, ruminants and swine while others such as rats are found to recruit them (reviewed in (Sasmono and Hume, 2004)) (Singh *et al.*, 1998). PIMs have also been referred to as pulmonary intravascular monocytes/macrophages (PIMMs) (Charavaryamath *et al.*, 2006) or pulmonary intravascular mononuclear phagocytes (PIMP) (Singh *et al.*, 1998). The lung macrophages are derived either from blood monocytes or through local replication in response to colony stimulating factor (reviewed in (Sasmono and Hume, 2004)).

1.7.1. Pulmonary intravascular macrophages (PIMs)

PIMs are a recently identified cell population described in few domestic animal species (Brain *et al.*, 1999) and cetaceans (Kawashima *et al.*, 2004). While they are resident macrophages in some species (equine, ruminants and swine), they are recruited in rats following bile duct ligation (Chang and Ohara, 1994), sepsis (Singh *et al.*, 1998) and an exposure to swine barn air (Gamage *et al.*, 2007). Interestingly, human patients undergoing thoracotomy for the excision of non-infectious disease have shown recruitment of PIMs (Dehring and Wismar, 1989).

PIMs are larger in size, metabolically more active than other lung macrophages and are tightly adhered to endothelium making their isolation from the lung tissue difficult (Rogers *et al.*, 1994). PIMs are highly phagocytic to bacteria, LPS and other injected tracer particles (Warner, 1996; Warner *et al.*, 1987; Singh and Atwal, 1997; Warner *et al.*, 1986) and are known to engulf red blood cells, neutrophils, fibrin and cell debris to assist in the resolution of inflammation (Atwal and Saldanha, 1985; Bertram, 1986; Warner *et al.*, 1987; Schneeberger-Keeley and Burger, Jr., 1970) and are involved in antigen presentation (Chitko-McKown *et al.*, 1991).

1.7.2. PIMs as resident macrophages

Species with resident PIMs retain intravenously injected tracer particles and endotoxin and exhibit pulmonary hypertension and lung microvascular leakage (Sone *et al.*, 1999) (reviewed in (Longworth, 1997)). Further, species with resident PIMs such as horses, show heightened sensitivity to endotoxemia (Longworth, 1997). This is interesting because PIMs express TLR4 and show direct colocalization of TLR4 and LPS both in their cytoplasm and nucleus (Wassef *et al.*, 2004; Singh *et al.*, 2006). In a number of experiments, PIMs have shown to be activated by endotoxin, bacteria and viruses to produce higher amounts of pro-inflammatory cytokines and the mechanisms of activation remain unresolved. Following LPS challenge, PIMs enlarge in size and up-regulate the expression of TNF-α, IL-1β, IL-6, IL-8, and COX-2 (Chen *et al.*, 2003). Resident PIMs produce increased amounts of pro-inflammatory cytokines and other mediators, they possibly activate the lung microvascular endothelium. Furthermore, PIMs have been shown to promote recruitment of inflammatory cells such as neutrophils and IL-8-containing platelets (Carrasco *et al.*, 2002; Singh *et al.*, 2004). Therefore species with resident PIMs are prone to suffer robust acute lung inflammation due to endotoxemia (Staub, 1994; Miyamoto *et al.*, 1988; Sone *et al.*, 1999; Staub *et al.*, 2001; Singh and de la Concha-Bermejillo, 1998).

Resident PIMs cause increased sensitivity for acute lung inflammation (ALI) and depletion of PIMs either using gadolinium chloride (GC) or liposomal chlodronate has shown to reduce the severity of lung inflammation. For example, depletion of PIMs using liposomal chlodronate reduced TNF-α, IL-6 and thrombin levels in swine endotoxemia model preventing ALI (Gaca *et al.*, 2003). Depletion of PIMs using GC has been shown to abrogate lung inflammation in calf and horse models of ALI through down-regulation of pro-inflammatory cytokines and reduction in the number of inflammatory cells (Singh *et al.*, 2004; Parbhakar *et al.*, 2005). Taken together, these data demonstrate a pro-inflammatory role for PIMs in acute lung inflammation.

1.7.3. Recruitment and functions of PIMs

Resident PIMs when stimulated produce pro-inflammatory cytokines and promote acute lung inflammation (Singh *et al.*, 2004; Parbhakar *et al.*, 2005). On the contrary, species that lack resident PIMs such as rats and mice display comparative resistance to endotoxin-induced acute lung inflammation. However, certain conditions such as sepsis (Singh *et al.*, 1998) or bile duct ligation (Chang and Ohara, 1994) or a single exposure to swine barn air (Gamage *et al.*, 2007) have been shown to recruit PIMs in rats. Rats subjected to bile duct ligation-induced cirrhosis recruited PIMs and showed unexpected lung uptake of adenovirus vectors and 60% mortality when compared to 11% deaths in controls (Smith *et al.*, 2004b). Following recruitment of PIMs and vector delivery, rats showed lethal hemorrhagic edema in the lung and increased levels of TNF-α and IL-6 in lung and serum. However, depletion of recruited PIMs from cirrhotic rats using GC has proven beneficial in reducing lung inflammation (Gill, 2005). Similar to cirrhotic rats with recruited PIMs, human patients with cirrhosis who receive the adenovirus vector are at risk of increased lung damage. Therefore, detailed investigation of functions of recruited PIMs is necessary. Our recent work in a rat model of swine barn air induced lung inflammation showed recruitment of PIMs and robust lung inflammation following a secondary LPS challenge. Interestingly, these recruited PIMs in the lungs of barn exposed rats showed the expression of TNF-α, IL-1β and TGF-β to indicate their capacity to produce multiple cytokines and influence the outcome of lung inflammation (Gamage *et al.*, 2007). Taken together, these data show PIM recruitment in species that are normally devoid of them and that recruited PIMs may increase susceptibility for acute lung inflammation. However, complete understanding of recruitment and functions of PIMs in models of sepsis is critically lacking.

1.7.4. Alveolar macrophages and lung inflammation

Alveolar macrophages are in direct contact with the inhaled air and act as the first line of defense (Monick and Hunninghake, 2003). They form 95% of the cells of the lung lavage fluid with the remainder portion comprised mostly of leukocytes. Alveolar macrophages express a number of receptors (reviewed in (Gordon and Read, 2002))

including TLR4 and other TLRs (Droemann *et al.*, 2003; Droemann *et al.*, 2005) and are involved in sensing endotoxin and other microbes. When alveolar macrophages encounter LPS, they get activated through TLR4-NF-κB pathway to produce pro-inflammatory cytokines, reactive oxygen and nitrogen mediators, enzymes, plasma proteins, growth factors and lipid mediators to influence the inflammatory process (Fujiwara and Kobayashi, 2005). Alveolar macrophages also produce IL-6, TGF-β, fibroblast growth factor, platelet derived growth factors and colony stimulating factors. Alveolar macrophages could produce many chemotactic factors such as IL-8, macrophage inflammatory proteins 1 and 2 (MIP-1 and MIP-2), leukotriene B4 (LTB4) and PDGF to attract granulocytes. Along with neutrophils, macrophages are involved in the phagocytosis and killing of bacteria, aerogenic fungi and some of the respiratory viruses (Lohmann-Matthes *et al.*, 1994).

1.7.5. Interstitial macrophages and lung inflammation

Interstitial macrophages are located in the connective tissue compartment. They are smaller, resemble blood monocytes and are less phagocytic when compared to other lung macrophages. However when compared to alveolar macrophages, interstitial macrophages have increased expression of MHC class II molecules to perform specific immune functions. Interstitial macrophages are also involved in producing inflammatory cytokines, oxygen and nitrogen free radicals and many other macrophage products (Lohmann-Matthes *et al.*, 1994; Sasmono and Hume, 2004).

1.8. Neutrophils and lung inflammation

Neutrophils are important components of the innate immune system and account for about 40-70% of the total leukocytes under normal homeostasis (Kuijpers and Roos, 2004). Neutrophils are produced in the bone marrow and circulate in the blood for about six hours during which they may migrate to the site of injury or undergo programmed cell death in the spleen followed by phagocytosis by macrophages. Each mature neutrophil measures about 10-15 μm in diameter with a segmented nucleus. The cytoplasm of the neutrophil contains specific and azurophilic granules. The specific granules are major

ones and contain lysozyme, collagenase and elastase while the azurophilic granules contain lysozymes and other microbicidal molecules (Abbas and Lichtman, 2005b).

Neutrophils are predominantly recruited into the lung in response to endotoxin (Snella and Rylander, 1982; Pauwels *et al.*, 1990; Harmsen, 1988). Neutrophil recruitment is a complex and multi-step process involving tethering, rolling of neutrophils on endothelium, adhesion, intraluminal crawling and transmigration. This process involves expression of selectins (E-selectin, P-selectin and L-selectin), integrins and their ligands (immunoglobulin superfamily members), chemokines, adhesion molecules and many other molecules (Ley *et al.*, 2007; Kelly *et al.*, 2007). Neutrophils express TLR2, TLR4, CD14 (Kurt-Jones *et al.*, 2002; Andonegui *et al.*, 2003) and other surface receptors to recognize microbes. Activated neutrophils produce reactive oxygen intermediates as well as contain myeloperoxidase, cathepsins, lysozyme, elastase, proteinase and other enzymes. These products of neutrophils kill bacteria but also damage lung cells (Reed and Milton, 2001; Sigsgaard *et al.*, 1994). Neutrophils secrete a number of important proteins involved in innate inflammatory process. For example, neutrophils produce a number of chemokines such as IL-8, GRO-α, MIP-1α, MIP-1β and cytokines namely such as IL-1β and TNF-α (Strieter *et al.*, 1992; Gasperini *et al.*, 1995; Kasama *et al.*, 1994; Bazzoni *et al.*, 1991; Lloyd and Oppenheim, 1992; Borregaard *et al.*, 2007). Taken together, neutrophils are a source of many microbicidal enzymes and produce many chemokines and cytokines to play a central role in innate recognition and microbial killing via phagocytosis and also influence the innate inflammatory process.

1.9. Cytokines in endotoxin induced lung inflammation

Cytokines are a group of small polypeptides or glycoproteins elaborated by the host cells with pleiotropic and redundant actions. The network of many cytokines together orchestrate the innate immune responses through interactions among themselves and binding to the specific receptors on the target cells (Abbas and Lichtman, 2005a; Tosi, 2005).

In endotoxin-induced lung inflammation, monocytes/macrophages are the main sources of key pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Reed and

Milton, 2001; Fujiwara and Kobayashi, 2005; Beutler and Kruys, 1995; Strieter *et al.*, 2002; Strieter *et al.*, 2003). TNF-α is one of the early response cytokines, originally purified from a conditioned medium of an LPS-stimulated macrophage cell line (Beutler *et al.*, 1985a). TNF-α is secreted as propeptide (26 kDa) which cleaves to yield an active molecule (17 kDa). TNF-α has two receptors, namely TNFR1 (55 kDa) and TNFR2 (75 kDa) (Bazzoni and Beutler, 1996). Binding to TNFR1 induces most of its biological effects (Vandenabeele *et al.*, 1995). TNF-α levels are increased early in a variety of humans and animal models of pneumonia (Laichalk *et al.*, 1996; Brieland *et al.*, 1995; Mehrad *et al.*, 1999) and TNF-α influences neutrophil recruitment through its regulation of expression of adhesion molecules and chemokines (Laichalk *et al.*, 1996). TNF-α in low levels increases phagocytosis and microbial killing by macrophages while higher levels result in tissue damage (Thacker, 2006). In general, TNF-α mimics LPS and induces fever, diarrhea, shock and death while antibody-mediated blocking of TNF-α reduces LPS-induced effects significantly (Beutler *et al.*, 1985b). Excessive amounts of TNF-α damage the host tissue, while complete blockade of TNF-α impairs bacterial clearance (Mehrad *et al.*, 1999). Therefore, a regulated expression of TNF-α is very important for host response to infection.

The IL-1 cytokine family consists of IL-1α and IL-1β (agonists), IL-1 receptor antagonist (IL-1ra) (antagonist) and two receptors (one signaling and one non-signaling) (Dinarello, 1998). IL-1α shares many properties with TNF-α such as its production is strongly induced by LPS and the chief cellular sources of IL-1α are monocytes and macrophages (Tosi, 2005). Further, IL-1α induces leukocyte adhesion to the endothelial cells (Strieter *et al.*, 2002; Thacker, 2006), IL-6 production (Cohen, 2002) and fever (Abbas and Lichtman, 2005a). However, unlike TNF-α, IL-1α is not cytotoxic (Abbas and Lichtman, 2005a). Both TNF-α and IL-1α have several soluble antagonists such as TNF-α receptor, IL-1ra, IL-10 and IL-6 secretion (Cohen, 2002; Feghali and Wright, 1997).

IL-6 is another pro-inflammatory cytokine elaborated by a variety of cells including monocytes, macrophages, T cells and fibroblasts (Van, 1990; Hirano, 1992).

IL-6 induces production of acute phase proteins, B cell maturation, T cell activation and provides negative feedback for TNF-α production (Feghali and Wright, 1997).

Pro-inflammatory cytokines are involved in initiation of an inflammatory event. Another set of cytokines control the actions of pro-inflammatory cytokines, down regulate the inflammatory process and assist in the healing process; cytokines in this group are termed, anti-inflammatory cytokines. Currently, TGF-β, IL-4, IL-6, IL-9, IL-10, IL-11, IL-13, IL-1ra, cytokine receptors for IL-1, TNF-α and IL-18 are considered as anti-inflammatory cytokines. IL-10 is the most potent anti-inflammatory cytokine (de Vries, 1995; de Waal *et al.*, 1992). IL-10 is mostly synthesized by CD4+Th0, CD4+Th1, CD4+Th2 cells, monocytes, macrophages, B cells, epithelial cells and tumor cells (Opal and DePalo, 2000b; Standiford, 2000). IL-10 strongly inhibits secretion of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 secretion from neutrophils, eosinophils and monocytes/macrophages (Brandtzaeg *et al.*, 1996; Clarke *et al.*, 1998; Gerard *et al.*, 1993; Marchant *et al.*, 1994). IL-10 blocks the nuclear translocation of NF-κB following LPS stimulation and also degrades pro-inflammatory cytokine mRNAs (Opal *et al.*, 1998). Mice lacking the IL-10 gene show heightened sensitivity to endotoxin-induced shock (Dai *et al.*, 1997) and IL-10 knockout mice spontaneously develop chronic inflammatory enteritis to underscore the importance of IL-10 in tightly regulating the inflammatory process (Kuhn *et al.*, 1993). Furthermore, IL-10 treatment has been shown to reduce the lethal effects of sepsis and is considered as a candidate for immunotherapy in septic patients (reviewed in (Standiford, 2000)).

Transforming Growth Factor-β (TGF-β) has three isoforms (TGF-β1, β2 and β3) all of which are encoded by separate genes but bind to the common receptor (Border and Ruoslahti, 1992). TGF-β is secreted by lymphocytes, macrophages and dendritic cells and regulates cell proliferation, differentiation and formation of extra-cellular matrix (Letterio and Roberts, 1998; Branton and Kopp, 1999). TGF-β could modulate the expression of adhesion molecules and inhibit the activated inflammatory cells (Letterio and Roberts, 1998). TGF-β signaling is mediated through Smad family proteins whose phosphorylation by TGF-β and subsequent translocation to the nucleus increases the transcription of target genes (Massague, 1998). TGF-β plays a pivotal role in tissue repair

following injury through reduction of pro-inflammatory cytokine production, enhancing the recruitment of fibroblasts, differentiation of myofibroblasts and production of extra-cellular matrix components (Lasky and Brody, 2000). Therefore, TGF-β has the ability to transform the inflammatory process at a local site into the resolution phase and assist in the repair of the damaged tissue (Letterio and Roberts, 1997). Furthermore, its importance is evident by the fact that mice lacking TGF-β1 gene show severe and uncontrolled inflammation that eventually leads to death (Shull *et al.*, 1992).

Once the inflammatory process eliminates the etiology, usually resolution occurs without detectable host tissue damage. However, when the stimulus is continuous or factors that facilitate resolution are not effective, chronic inflammation may result. One of the first steps in resolution is taming the activated inflammatory cells and neutralizing their effect. Recruited leukocytes after completing their job, re-enter the circulation (Hughes *et al.*, 1997; Martin *et al.*, 2003), or enter the lymphatic drainage (Bellingen *et al.*, 1996; Bellingan *et al.*, 2002) or die either by apoptosis or necrosis and subsequently are engulfed by macrophages (Ishii *et al.*, 1998). Macrophages following ingestion of effete neutrophils secrete TGF-β1 and hepatocyte growth factor. Both TGF-β1 and hepatocyte growth factor are important in the resolution of lung inflammation and hepatocyte growth factor has shown to induce DNA synthesis in alovelar type-II cells (Mason *et al.*, 1994; Shiratori *et al.*, 1995). Re-epithelization of the bronchial and alveolar region is critical for the removal of the edema fluid for efficient gas-change. Many other growth factors present in the edema fluid themselves have been shown to promote the alveolar epithelial repair *in vitro* (Geiser, 2003). Macrophages with ingested neutrophils are also removed either by apoptosis or lymphatic drainage. The process of resolution and tissue repair is complex involving several mediators and cells. The efficiency of resolution and repair determines the extent of reversal to the original functional status of the tissue and organ while failure will result in excessive tissue scarring, loss of original structure and function and onset of the chronic inflammatory process.

1.9.1. Summary and conclusions

The innate immune mechanisms in the lung are highly efficient and hence infections of the respiratory tract are rare despite its exposure to high volume of diverse antigens, chemicals, biological and physical materials. Innate inflammatory response removes the invading microbes and their products to nullify invasion. If the pathogen load is higher, antigen specific adaptive immunity begins and helps the body fight exposure. The normal inflammatory processes usually resolve quickly without detectable damage to the host. However, failure to resolve and constant invasion of pathogens could result in chronic inflammation. Although there is emerging consensus on the basic mechanisms of lung inflammation induced following exposure to endotoxins, we know little about the innate mechanisms of lung inflammation induced by exposure to complex air in the pig barns.

1.9.2. Rationale for the experiments conducted

In the following sections, I will discuss the rationale for conducting the experiments contained in this thesis. I will also summarize the same in the form of a flow chart (Figure 1.1). The primary focus of my research is to understand the mechanisms of lung inflammation and lung dysfunction following exposure to swine barn air. Because of the emerging role of PIMMs, I also studied the recruitment and functions of PIMMs in sepsis-induced lung inflammation. PIMMs are present in the lungs of few domestic animal species as a resident population (Atwal *et al.*, 1992; Staub, 1994) and have been linked to the increased susceptibility of the host to endotoxemia (Longworth, 1997). Species such as rats, normally devoid of PIMMs, recruit them with *E. coli* induced sepsis (Singh *et al.*, 1998) or a single exposure to the barn air (Gamage *et al.*, 2007). PIMM recruitment has been shown to peak at 48 hour post-barn exposure and a secondary challenge with *E. coli* LPS at this time point has been shown to result in robust lung inflammation (Gamage *et al.*, 2007). However, it is unclear if septic rats with recruited PIMMs in their lungs would have similar heightened susceptibility to a secondary challenge with *E. coli* LPS. Therefore, I designed my first experiment with an objective of characterizing the recruitment and functions of PIMMs in a rat model of sepsis and a secondary challenge with *E. coli* LPS.

With an understanding of the recruitment and functions of PIMMs in rat models of sepsis and swine barn air induced lung inflammation, we decided to investigate the mechanisms of lung inflammation following swine barn exposure in detail. Although experimental single exposure of naïve human volunteers have documented induction of lung inflammation with increased AHR, the cell and molecular mechanisms of this remained unresolved (Wang *et al.*, 1997; Palmberg *et al.*, 2002; Larsson *et al.*, 1994; Dosman *et al.*, 2000; Palmberg *et al.*, 2004). Other than a report describing chronic exposure of rabbits and guinea pigs to the barn air, there have been no studies with animal models (Donham and Leininger, 1984). Therefore, first, I decided to characterize a rat model to use for investigations related to the mechanisms of lung dysfunction following exposure to the barn air. My model mimics a pattern of exposure to a full time barn worker, with experimental barn exposure of rats for eight hours per day for one or five days or four cycles of five days. My treatment groups will simulate naïve workers exposure following the first day in the barn, exposure effects after one work week (five days) and exposure effects following four work weeks inside the barn respectively. Following exposure to the barn or ambient air (controls), measurement of AHR, analysis of blood, BALF and lung tissue will investigate the *in situ* cell and molecular mechanisms of lung dysfunction following single and multiple exposures to swine barn air.

The swine barn environment is a heterogeneous mixture of many injurious agents (Vogelzang *et al.*, 1998; Donham and Popendorf, 1985; Asmar *et al.*, 2001) known to affect exposed individuals (reviewed in (Charavaryamath and Singh, 2006)). However endotoxin concentration in the barn environment appears to be the prime factor in causing many negative health effects (Vogelzang *et al.*, 1998). Therefore, in order to clarify the role of endotoxin, I exploited the fact that TLR4 is the cellular receptor for endotoxin (Takeda *et al.*, 2003; Aderem and Ulevitch, 2000) and mutations in TLR4 result in a blunted response to endotoxin or LPS (Arbour *et al.*, 2000; Schwartz, 2001). Therefore, using C3HeB/FeJ (wild type [WT], functional TLR4) and C3H/HeJ mice (mutant, natural point mutation in *TLR4* gene), I performed an experiment similar to my rat model of occupational exposure to the swine barn air. I measured AHR and assessed lung inflammation to clarify the role of endotoxin in inducing them.

My fourth experiment was aimed at investigating the susceptibility of rats to *E. coli* LPS following single and five day barn exposures. Previously, we have shown that, following single eight-hour barn exposure, rats will recruit PIMs and show robust lung inflammation with a secondary *E. coli* LPS challenge at 48 hour post-barn exposure (Gamage *et al.*, 2007). I examined the effect of single or multiple exposures to the barn air and a secondary challenge with *E. coli* LPS.

Following our investigation into the role of endotoxin in inducing AHR and lung inflammation, as well as lung responses to endotoxin following barn exposure, I decided to explore the possibility of involvement of other cell signaling molecules. Although there are many signaling molecules, I focused on calcineurin (CaN) and *N*-myristoyltransferase (NMT), because of their roles in TLR4 signaling and inflammation (Rusnak and Mertz, 2000; Macian *et al.*, 2001; Bueno *et al.*, 2002; Fernandez *et al.*, 2007; Kim *et al.*, 2004b; Kang *et al.*, 2007; Selvakumar *et al.*, 2007; Sharma, 2004; Magnuson *et al.*, 1995; Price *et al.*, 2003; Shrivastav *et al.*, 2005; Shrivastav *et al.*, 2007; Rowe *et al.*, 2006). I examined the expression and activity of NMT and CaN in lung tissues from animals exposed to the barn air as well as challenge with *E. coli* LPS.

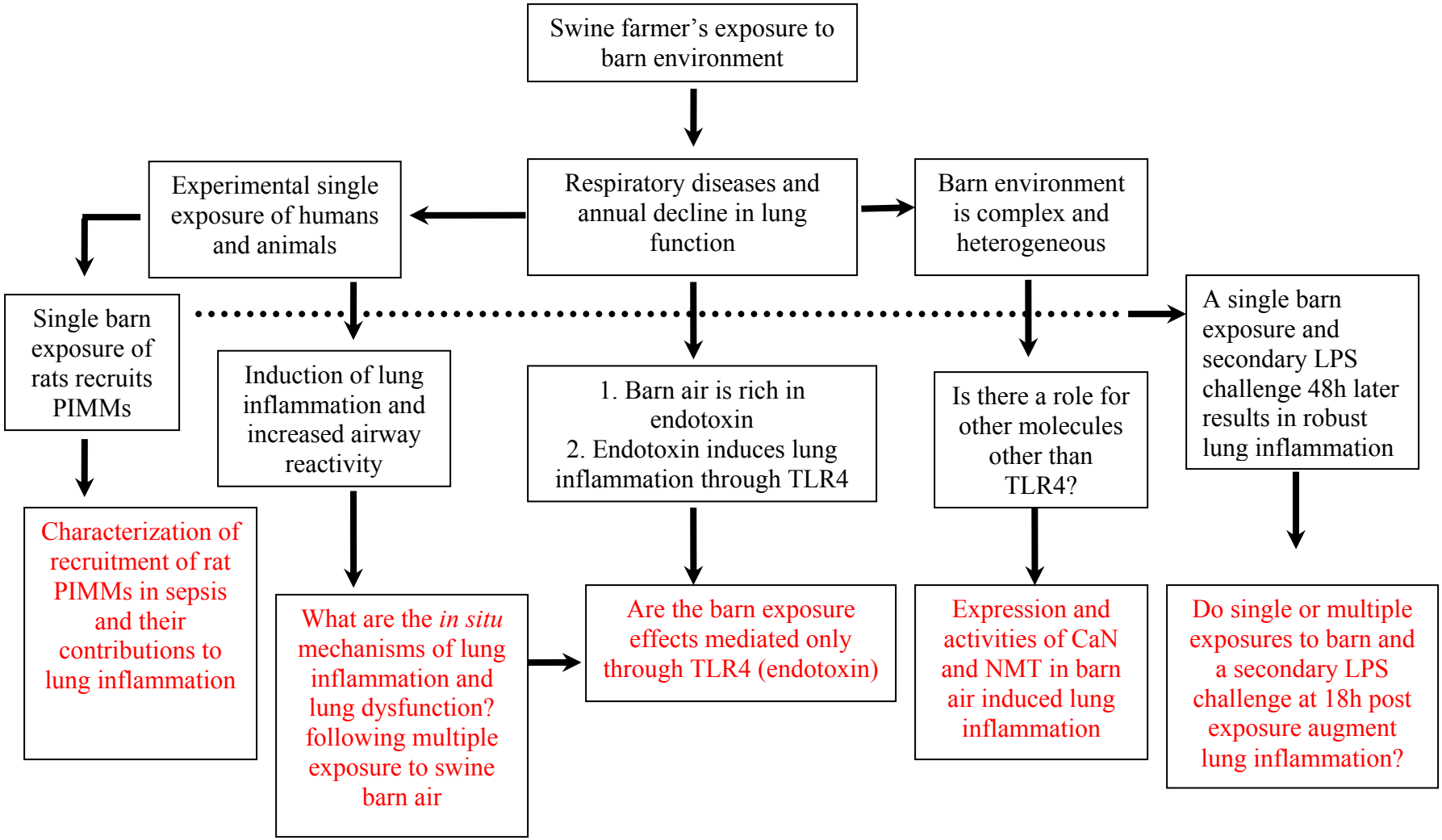

Figure 1.2. Summary of rationale of experiments.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1. Hypotheses

1. *E. coli*-induced sepsis induces recruitment of PIMMs in rat lungs and a secondary challenge with *E. coli* LPS at 48 hour later enhances lung inflammation in rats.
2. Multiple exposures to swine barn air induce lung inflammation and a decline in lung function.
3. Lung inflammation and AHR following single or multiple exposures to endotoxin-rich barn air are mediated through an intact TLR4-pathway.
4. Single or multiple exposures to swine barn air increase the susceptibility for lung inflammation in response to a secondary *E. coli* LPS challenge.
5. Barn exposure induces lung inflammation as well as increases expression and activity of NMT and CaN in rat lungs.

2.2. Objectives

1. To characterize recruitment and functions of PIMMs in a rat model of sepsis.
2. To evaluate the effect of single and multiple exposures to swine barn air.
3. To evaluate the role of TLR4 in inducing lung dysfunction following exposure to swine barn air.
4. To evaluate effect of a secondary challenge with *E. coli* LPS in one or five day swine barn exposed rats.
5. To characterize the expression and activities of CaN and NMT in lung inflammation induced following either exposure to swine barn air or *E. coli* LPS.

CHAPTER 3: PULMONARY INTRAVASCULAR MONOCYTES/MACROPHAGES IN A RAT MODEL OF SEPSIS *

3.1. Abstract

Sepsis induces recruitment of neutrophils and monocytes/macrophages in the lung and enhances host susceptibility to a secondary bacterial challenge. The phenotype and functions of recruited pulmonary intravascular monocytes/macrophages (PIMMs) in sepsis remain largely unknown. Therefore, we characterized PIMM recruitment and functions in a rat model of *E. coli*-induced sepsis. Male Sprague-Dawley rats were injected intraperitoneally with saline (N=10) and 48 hours after the saline treatment treated intravenously with either saline (n=5) or *E. coli* lipopolysachharide (LPS) (1.5 μg/kg body weight; n=5). Second group of 10 rats was infected intraperitoneally with *E. coli* ($2X10^7$ CFU/100 g) followed by intravenous injection of either saline (n=5) or the LPS (n=5) 48 hours after the first treatment. Rats were euthanized at 6 hours after the LPS treatment. Immunocytochemistry showed more PIMMs stained with ED-1 antibody, which specifically reacts with rat monocytes/macrophages, in rats infected with *E. coli* compared with the controls ($P<0.05$). The LPS treatment of *E. coli* infected rats increased the numbers of PIMMs ($P<0.05$) and induced more inflammation compared to other groups. Immuno-electron microscopy localized TNF-α, IL-10 and TGF-β2 in recruited PIMMs in rats challenged with both *E. coli* and the LPS. ELISA on lung homogenates showed higher concentrations of TNF-α, IL-10 and TGF-β2 in rats treated with both *E. coli* and LPS compared with those treated with only LPS or *E. coli* ($P<0.05$). We conclude that ED-1 positive PIMMs are recruited in this model of sepsis and contain TNF-α, IL-10, and TGF-β2.

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http://www3.interscience.wiley.com/cgi-bin/fulltext/113446033/PDFSTART

3.2. Introduction

Sepsis is a complex clinical syndrome seen as a result of a systemic inflammatory response to live bacteria or bacterial products (Villar *et al.*, 2004).Sepsis induces lung inflammation with alveolar recruitment of inflammatory cells such as neutrophils and monocytes/macrophages (Chignard and Balloy, 2000). Roles of neutrophils and monocytes/macrophages recruited into the alveoli in sepsis induced lung injury have been addressed by various studies (Ishii *et al.*, 1998; Cox *et al.*, 1995; Lomas-Neira *et al.*, 2005; Ellaban *et al.*, 2004). However, there is morphologic evidence for recruitment of pulmonary intravascular monocytes/macrophages (PIMMs) in a rat model of *E. coli*-induced sepsis (Singh *et al.*, 1998). Also, there is some evidence that marginated monocytes in the vasculature of endotoxemic mouse lung modulate the lung inflammation through TNF-α dependent up regulation of adhesion molecules on the endothelium (O'dea *et al.*, 2005). But the immuno-phenotype and functions of recruited PIMMs are not clearly understood.

Complications of sepsis could damage lung tissue and also predispose lungs to subsequent infections and pronounced lung injury (Welbourn and Young, 1992). Precise reasons for enhanced susceptibility for lung injury following a secondary microbial challenge are not well understood. Because activated monocytes and macrophages can produce inflammatory mediators (Fujiwara and Kobayashi, 2005), we speculated whether recruited PIMMs may contribute to increased susceptibility for lung inflammation following a secondary challenge in a rat model of sepsis. Therefore, the objectives of our study were to characterize recruitment of PIMMs in sepsis and their contributions to lung inflammation following a secondary challenge with *E. coli* LPS. Our data show that an intraperitoneal injection of *E. coli* induced recruitment of ED-1 positive PIMMs. Rats challenged with *E. coli* LPS following recruitment of PIMMs showed more intense lung inflammation and higher concentration of TNF-α, IL-10 and TGF-β2 compared to the LPS-treated control rats and localization of these cytokines in the PIMMs.

3.3. Materials and methods

3.3.1. Rats and treatment groups

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance in accordance with the guidelines of the Canadian Council on Animal Care. Specific pathogen-free, six to seven-week-old male Sprague-Dawley rats (~ 225 g body weight) were procured from Charles River laboratories, Canada. Rats were maintained in the animal care unit following acclimatization for a period of one week, with access to food and water *ad libitum* and light and dark cycle of 12 hours each. Rats were randomly divided into different groups (n=5/ group). All the personnel involved in evaluating the stained slides and interpreting results were blinded to the treatment groups.

Male Sprague-Dawley rats (N=20) were divided into two groups of ten rats each. One group was injected intraperitoneally with normal saline (N=10) followed by an intravenous injection of either saline (n=5) or *E. coli* LPS (n=5; O128: B12; Sigma-Aldrich; 1.5 µg/ kg of rat body weight) 48 hours after the first treatment. Second group of ten other rats was infected with *E. coli* (2 x10^7 CFU / 100g, intraperitoneal) and challenged intravenously with saline (n=5) or LPS (1.5 µg/kg body weight; n=5) similar to the first group. The rats were euthanized 6 hours after the second treatment because there is evidence that the LPS treatment induces lung inflammation within this time period (Janardhan *et al.*, 2006). Following a pre-determined approach, three pieces of tissue from each lung were collected for light microscopy by a person blinded to the treatments. The remainder of the lung tissues were used for immuno-electron microscopy or frozen for enzyme-linked immunosorbent assay (ELISA).

3.3.2. Hematoxyline-eosin staining and immunohistochemistry

Tissue pieces were fixed in 4% paraformaldehyde for 16 hours followed by three washes in phosphate buffered saline (PBS). Tissues were dehydrated in ascending concentrations of ethanol and xylene and embedded in paraffin. Five micrometer thick sections were cut and placed slides coated with 10% Poly-L-lysin (Sigma Diagnostics).

The slides with sections were kept in an oven (40 ^{o} C for 45 minutes) to improve the adherence of tissue sections followed by staining with hematoxyline and eosin.

Lung sections were processed for immunohistochemistry as described (Singh *et al.*, 2001). Briefly, the tissue sections were deparaffinized and rehydrated and incubated with 0.5% H_2O_2 in methanol for 20 minutes to quench endogenous peroxidase. After treating with pepsin (2mg /ml of 0.01 N HCl; 45 minutes) to unmask the antigens, the sections were blocked with 1% BSA (Albumin, Bovine; Sigma) and exposed to primary antibodies [TNF-α (1:75), von Willebrand Factor (1:300), TGF-β2 (1:200), IL-10 (1:400) and ED-1 (1: 200) all from Santa Cruz Biotechnology, except ED-1 (Serotec)]. The sections were incubated with appropriate biotinylated or horseradish peroxidase (HRP) conjugated secondary antibodies (1:150 and all from DakoCytomation Denmark). Whenever biotinylated secondary antibody was used, HRP conjugated streptavidin was added (1:3,000; DakoCytomation Denmark) before color development with a commercial kit (VECTOR, VIP; Vector laboratories, Burlingame, CA). Controls consisted of staining without primary antibody or isotype matched immunoglobulin instead of primary antibodies. Finally, tissue sections were counter stained using nuclear counter stain methyl green (Vector laboratories).

3.3.3. Immunohistochemical quantification of PIMMs

Sections obtained from one tissue block each from the right and left lung lobes from each rat were stained using ED-1 antibody, which recognizes monocytes/macrophages (Sminia and Dijkstra, 1986; Janardhan *et al.*, 2006; Mizgerd *et al.*, 1997). Following immunohistochemistry on lung sections, ED-1 positive cells in the lung septum were counted in ten high power fields (400X; 0.096 mm^2 per field) in each section and a total of 20 fields per rat. The fields for counting ED-1 positive cells were randomly selected and did not include larger blood vessels and airways.

3.3.4. Immuno-gold electron microscopy

Lungs samples were prepared for immuno-electron microscopy as described previously (Singh *et al.*, 2001). Briefly, tissues fixed in 0.1% gluteraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer for 3 hours at 4ºC, dehydrated and

infiltrated with LR White resins. The tissues were polymerized under ultraviolet light at -8 ºC for 3 days. Semithin (1 mm) sections were prepared to select areas for ultrathin (100 nm) sections. Sections were stained with ED-1 (1:100), IL-10 (1:200), TGF-β2 (1:25) and TNF-α (1: 25) antibodies followed by appropriate gold-conjugated secondary antibodies (respective, 1:100 diluted) and examined in an electron microscope at 60kV. Immuno-electron microscopy controls included omission of primary antibody or staining of lung sections with anti-von Willebrand Factor antibody.

3.3.5. ELISA

Concentration of TGF-β2, TNF-α, and IL-10 were measured using sandwich enzyme-linked immunosorbent assay employing commercial capture/detection antibody pairs and recombinant standards (IL-10 and TNF-α, BD Biosciences, Ontario,Canada and TGF-β2 from R&D Systems, MN), as described previously (Gordon *et al.*, 2000). Lung samples were homogenized in HBSS (0.1 g/ml) containing protease inhibitor cocktail (100 µl/10ml; Sigma-Aldrich, MO). Briefly, 96-well Immulon-4 HBX ELISA plates (Dynex Technologies, Chantilly, VA) were coated with capture antibody (over night at 4 ^{o}C), blocked using 1% bovine serum albumin (BSA, Sigma Aldrich, Canada), standards and samples (n=3; 100 µl each in duplicates) were added and plates were incubated at 37 ^{o}C for 2 hours, washed with PBS-Tween, detection antibody added and incubated (60 minutes at 37 ^{o}C). Final steps involved incubation with 1:3,000 dilution of streptavidin-conjugated horseradish peroxidase (Dako) and TMB Microwell Peroxidase Substrate System 2-C (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The assays were read at 450 nm and analyzed using an automated ELISA plate reader and the Microplate Manager software respectively (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

3.3.6. Statistical analysis

All data were expressed either as the mean ± SEM or median and inter-quartile range. Group differences were examined for significance using one-way analysis of variance with Fishers LSD as *post hoc* test or Kruskall-Wallis one-way analysis of variance with Dunn's comparison as the *post-hoc* test (Sigma Stat statistical software

version 2.0 for Windows 95,NT and 3.1, SPSS Inc., Chicago, IL). Statistical significance was established at $P<0.05$.

3.4. Results

3.4.1. PIMM recruitment

We characterized PIMM recruitment using immunohistochemistry and immuno-electron microscopy. The omission of primary antibody from the immunohistochemical protocol resulted in lack of staining in lung sections (Figure 3.1A) while incubation with anti-von Willebrand factor antibody delineated the lung vasculature (Figure 3.1B). Control rat lung sections showed fewer ED-1-positive septal monocytes/macrophages compared to those injected with *E. coli* followed by saline (Figure 3.1C). In contrast, lung sections from *E. coli*-infected rats showed many septal ED-1-positive monocytes/macrophages (Figure 3.1D). Immuno-gold electron microscopy confirmed that septal cells reactive for ED-1 antibody were in the lung microvessels and formed focal adhesions with the capillary endothelium (Figure 3.2). Some of the intravascular mononuclear phagocytes had ED-1 staining in their lysosomes (Figure 3.2, L-lysosome, inset). Therefore, based on the immunocytochemical data, we used ED-1 as a marker to quantify the PIMMs in normal and *E. coli*-infected rats. The data showed an increase in PIMM numbers in lungs of *E. coli*-infected group compared to the controls (Figure 3.1E; $P<0.05$).

3.4.2. Lung inflammation following secondary challenge with E. coli LPS

3.4.2.1. Histopathology

Lung sections from control rats showed no signs of inflammation indicated by the normal architecture (Figure 3.3A) while those treated with *E. coli* LPS exhibited congestion and infiltration of neutrophils and macrophages in the septa (Figure 3.3B, arrows). Lung tissues from rats infected with *E. coli* (Figure 3.3.C and D) showed inflammation including thick alveolar septa (arrowhead) and recruited monocytes/macrophages (Figure 3.3C, arrows) and neutrophils (Figure 3.3D, arrows and inset). It appeared that lungs from rats administered LPS after infection with *E. coli*

(Figure 3.3E) were the most inflamed with intense accumulation of neutrophils and macrophages in the septa (arrows and inset) and thickened septa (arrowheads). Compared with all other groups, rats challenged with both *E. coli* and *E. coli* LPS had more ED-1-positive PIMMs (Figure 3.3F; $P<0.05$). The rats given only the bacteria had higher numbers of PIMMs compared with those treated with saline only ($P<0.05$), while there were no differences between control and those treated with only LPS ($P>0.05$).

3.4.2.2. TNF-α expression and quantification

Immunohistology showed TNF-α-positive cells in the lung septum of all the groups (Figure 3.4A-D). TNF-α was also localized in bronchiolar epithelium and in some of the blood vessels (data not shown). TNF-α staining in the PIMMs was confirmed with ultrastructural immunocytochemistry (Figure 3.5). ELISA on lung homogenates from rats challenged with both *E. coli* and *E. coli* LPS showed higher concentrations of TNF-α compared to all other groups (Figure 3.4E; $P<0.05$). There were no differences between the rats treated with only the saline or the bacteria or LPS ($P>0.05$).

3.4.2.3. IL-10 expression and quantification

IL-10 staining was noticed in occasional septal cells in the lungs from the control rats (Figure 3.6A) but in many cells in the lungs from rats treated with LPS only (Figure 3.6B). Rats infected with *E. coli* bacteria followed by saline or LPS treatment showed increased intensity of IL-10 staining in the septa as well as in large septal cells (Figure 3.6C and D). Immuno-electron microscopy localized IL-10 in PIMMs (Figure 3.7). ELISA showed higher concentrations of IL-10 in rats treated with only saline or both the bacteria and LPS compared to the other two groups ($P<0.05$). Further, lung concentration of IL-10 was more in rats infected with *E. coli* than those treated with only LPS (Figure 3.6E; $P<0.05$).

3.4.2.4. TGF-β2 expression and quantification

Immunohistology showed only faint staining for TGF-β2 in lung sections from the control rats (Figure 3.8A). However, TGF-β2 staining was observed in many septal

cells in lungs from rats treated with the bacteria or LPS alone or both (Figure 3.8B-D). Immuno-electron microscopy showed TGF-β2 labeling in PIMMs (Figure 3.9). ELISA showed higher concentration of TGF-β2 in rats challenged with LPS following treatment with *E. coli* compared to all other groups (Figure 3.8E; $P<0.05$).

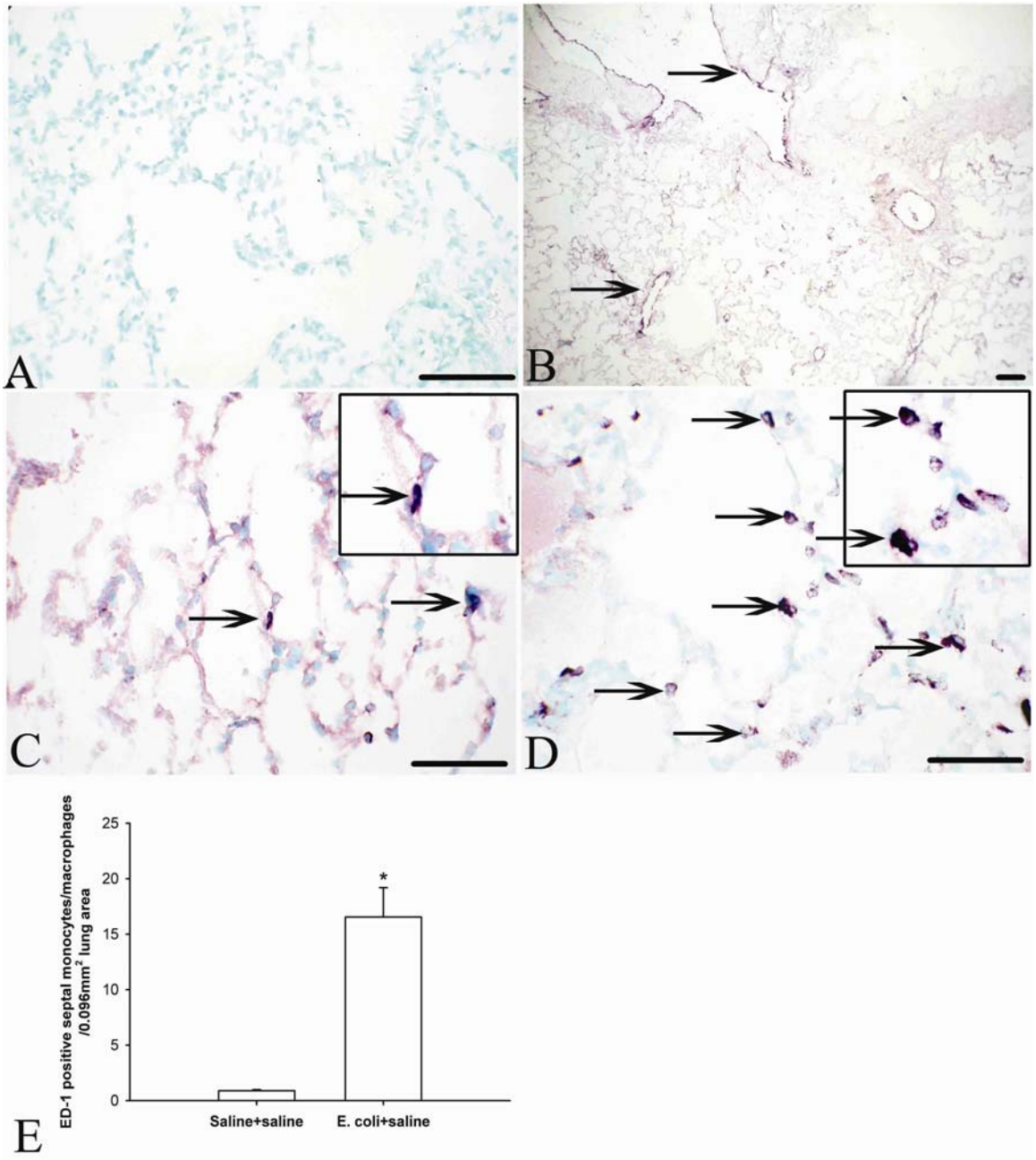

Figure 3.1. Recruitment of pulmonary intravascular monocytes/macrophages in the lung.

Omission of primary antibody (A) and use of anti-von Willebrand factor antibody (B) served as negative and positive controls, respectively. There were few ED-1 positive septal cells in controls (saline + saline; C and inset, arrows) while lungs from *E. coli* + saline-treated rats showed many ED-1 positive cells in the lung septum (D and inset, arrows). Compared to controls, rats treated with *E. coli* followed by saline showed a significant increase in ED-1 positive cells in the lung septa (E; asterisk, $P<0.05$). Original magnifications: 100X (B); 400X (A, C, and D). Scale bar= 50 μm

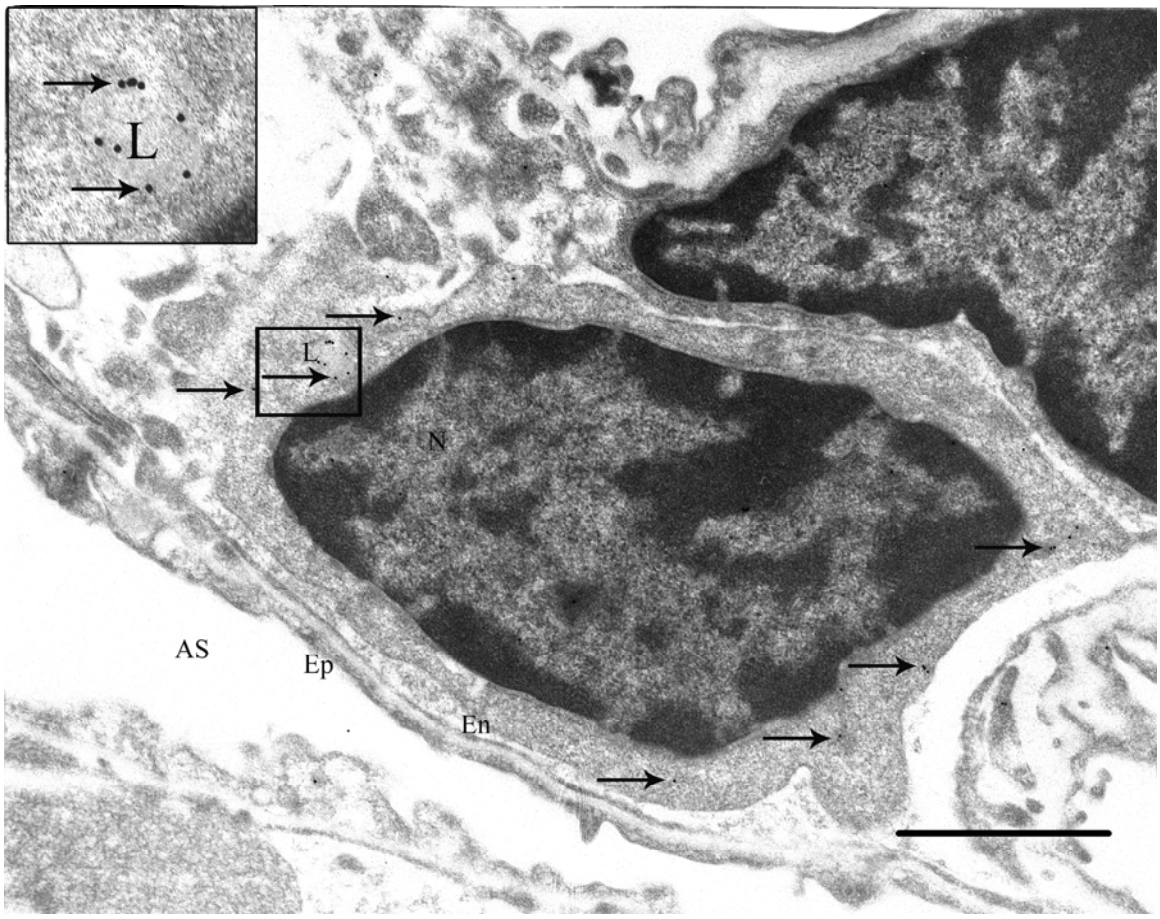

Figure 3.2. ED-1 immuno-electron microscopy.

A pulmonary intravascular monocyte/macrophage shows staining (arrows) with ED-1 antibody in cytoplasm and lysosomes (L; inset). N, nucleus; En, endothelium; Ep, epithelium; AS, alveolar space. Scale bar=1 μm. Magnification: 13,000 X.

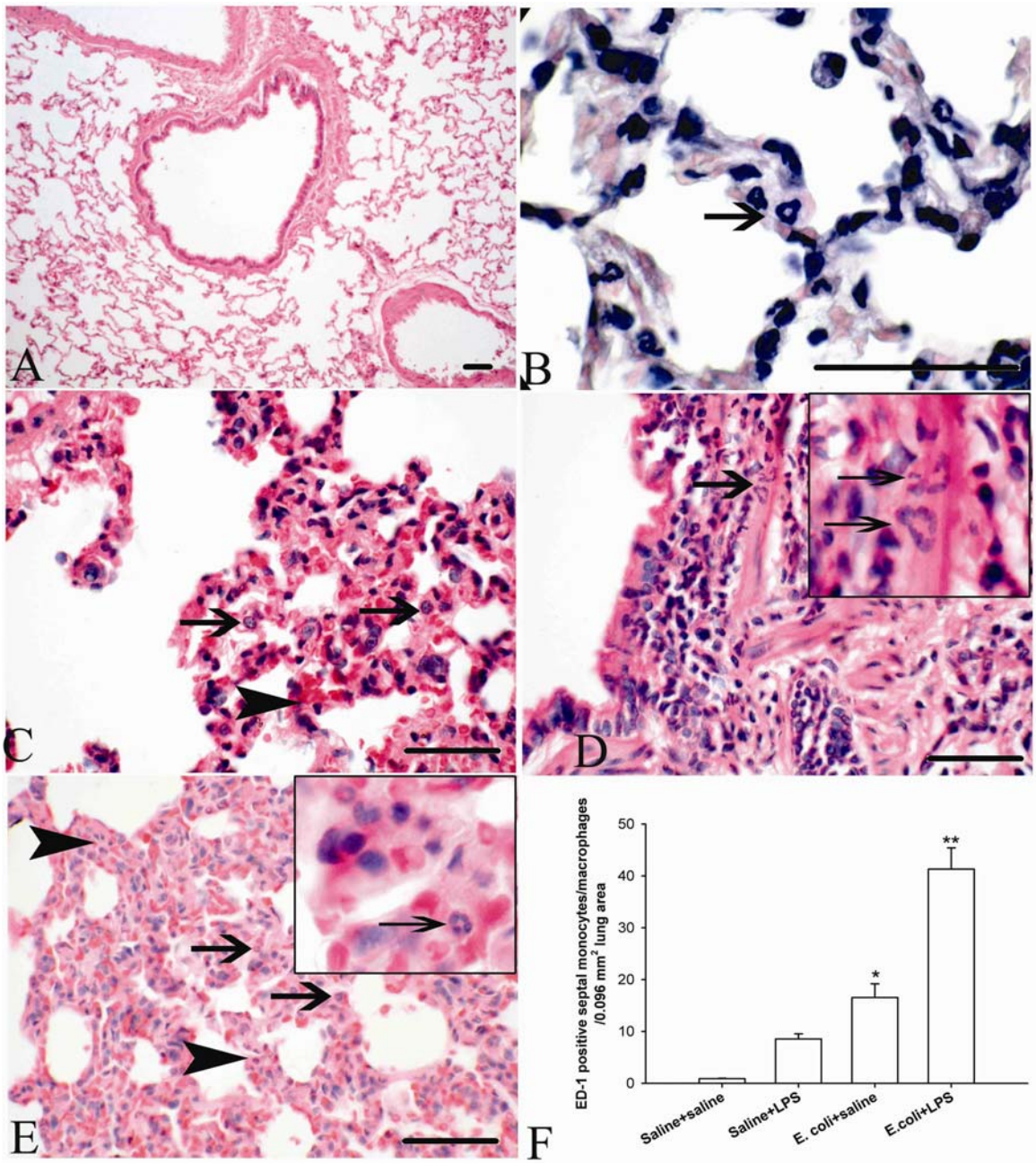

Figure 3.3. Lung inflammation.

Lung section from control rats (saline +saline; A) showed normal architecture and no signs of inflammation while those treated with either *E. coli* LPS (B) or *E. coli* bacteria (C and D) or both (E) showed septal thickening (arrowhead), recruitment of neutrophils (arrows in B, D, E and insets) and monocytes/macrophages (arrows in C). Rats treated with *E. coli* + *E. coli* LPS (E) showed more pronounced septal congestion (arrowhead). Figure F shows quantification of ED-1 positive cells in the septa in various groups. **: $P<0.05$ when compared to all other groups; asterisk, $P<0.05$ when compared to those treated with only the saline. Original magnification: 100X (A); 1,000X (B); 400X (C-E). Scale bar=50 μm.

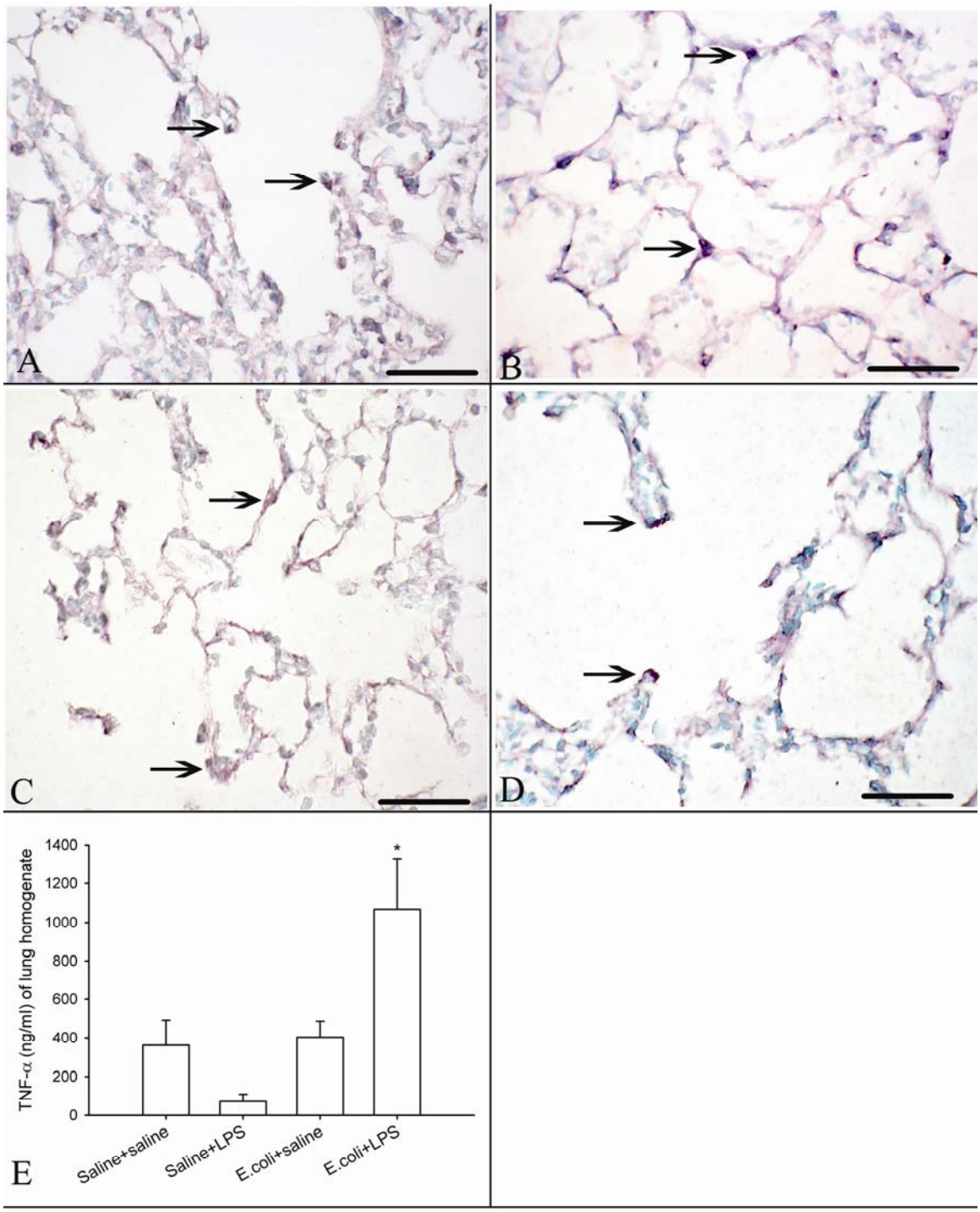

Figure 3.4. Expression of and quantification of TNF-α.

TNF-α positive septal cells (arrows) were observed in rats treated with saline + saline (A), saline + *E. coli* LPS (B), *E. coli* + saline (C) and *E. coli* + *E. coli* LPS (D). E. Quantification of TNF-α in the lung homogenates using ELISA. Asterisk, $P<0.05$ compared to other groups. Original magnification: 400X (A-D).Scale bar=50 µm.

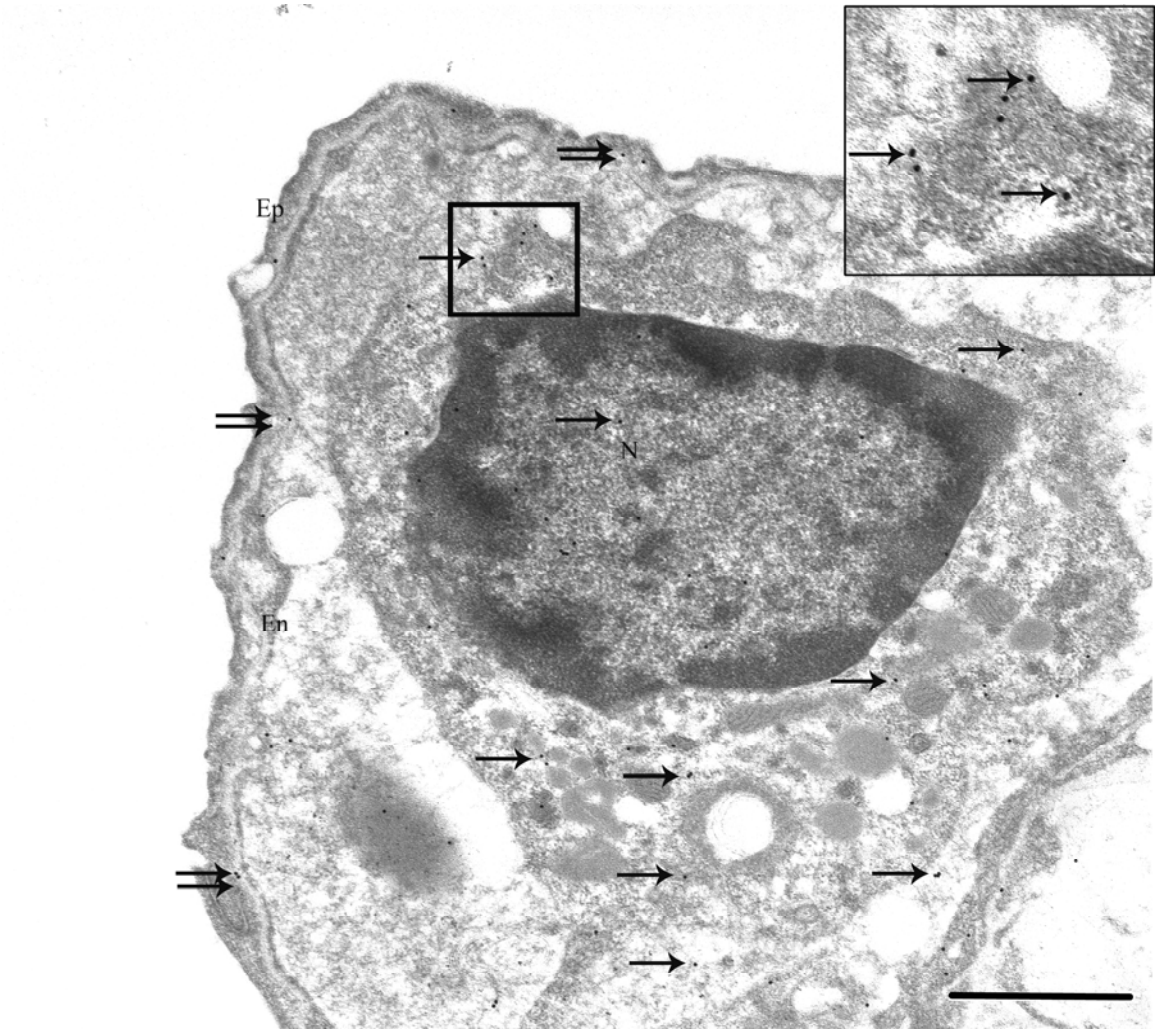

Figure 3.5. TNF-α immuno-electron microscopy.

A PIMM shows staining for TNF-α in the cytoplasm (single arrows and the inset) and the endothelium (double arrows). En, endothelium; Ep, epithelium. Scale bar=1 μm. Original magnification: 13,000X.

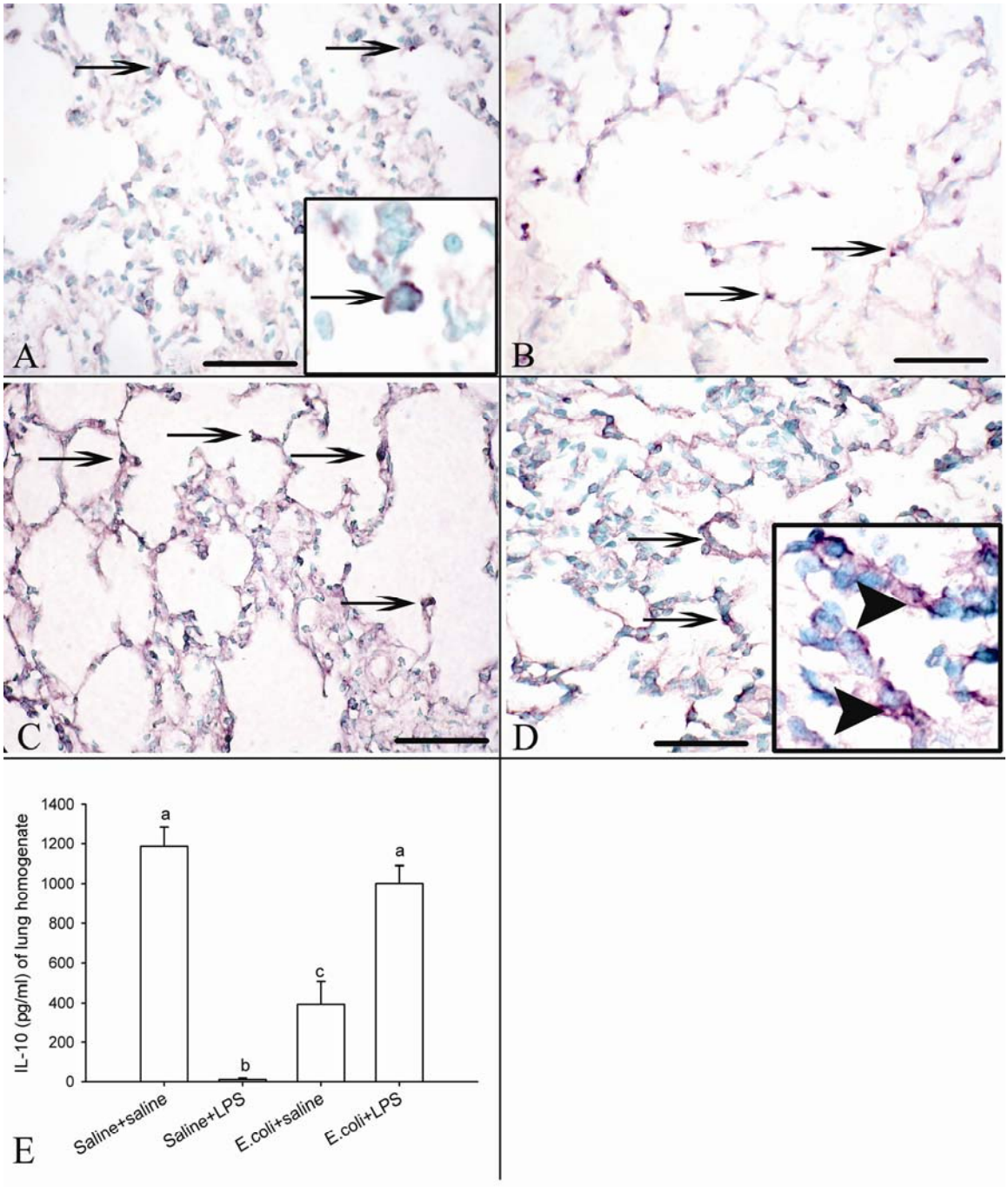

Figure 3.6. IL-10 expression in the lung.

IL-10 positive septal cells (arrows) were observed in rats treated with saline + saline (A), saline + *E. coli* LPS (B), *E. coli* + saline (C) and *E. coli* + *E. coli* LPS (D). Figure E shows quantification of IL-10 in the lung homogenates using ELISA. Groups bearing different superscripts differ significantly ($P<0.05$). Original magnification: 400X (A-D); 1,000X (insets). Scale bar=50 µm.

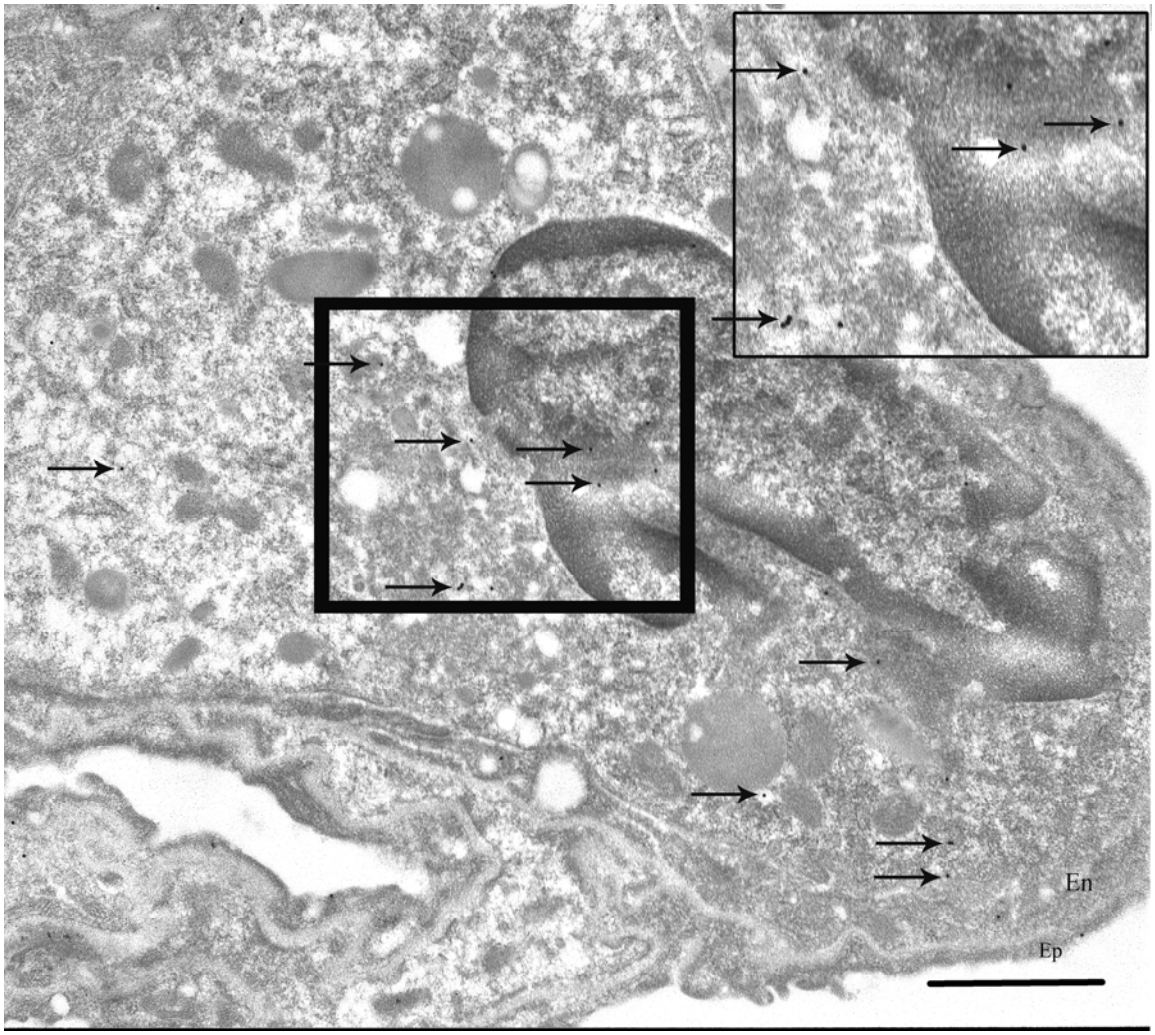

Figure 3.7. IL-10 immuno-electron microscopy.

IL-10 staining (arrows) is observed in the cytoplasm and the nucleus (inset) of a PIMM. En, endothelium; Ep, epithelium. Scale bar=1 μm. Original magnification: 13,000X.

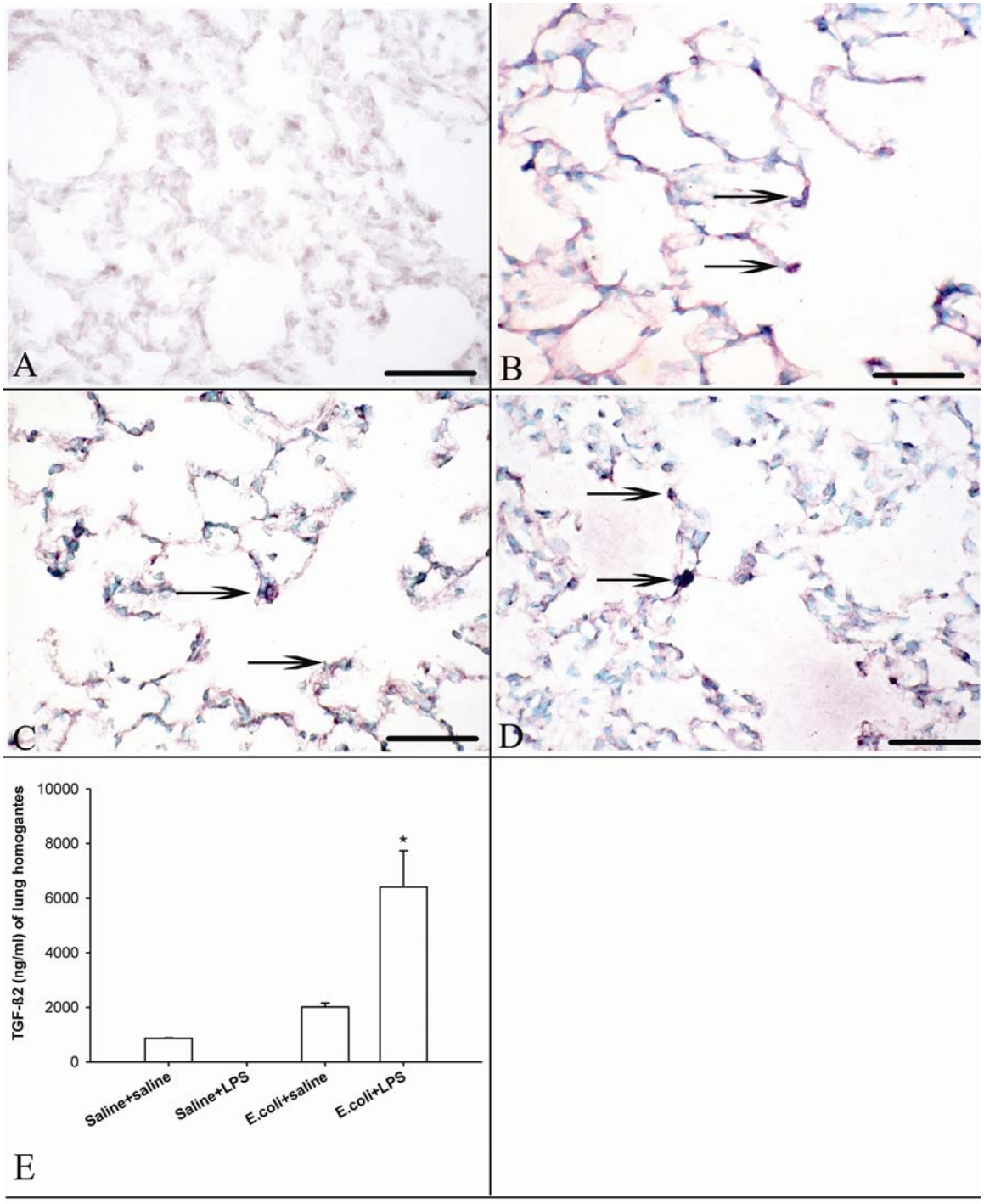

Figure 3.8. TGF-β2 expression in the lung.

Faint staining for TGF-β2 was seen in lung sections from rats treated with saline + saline (A) while TGF-β2 positive septal cells (arrows) were observed in those given *E. coli* LPS (B), *E. coli* (C) or both *E. coli* and *E. coli* LPS (D). E: Quantification of TGF-β2 in the lung homogenates by ELISA. Asterisk, significantly different from the other three groups ($P<0.05$). Original magnification: 400X (A-D). Scale bar =50 µm.

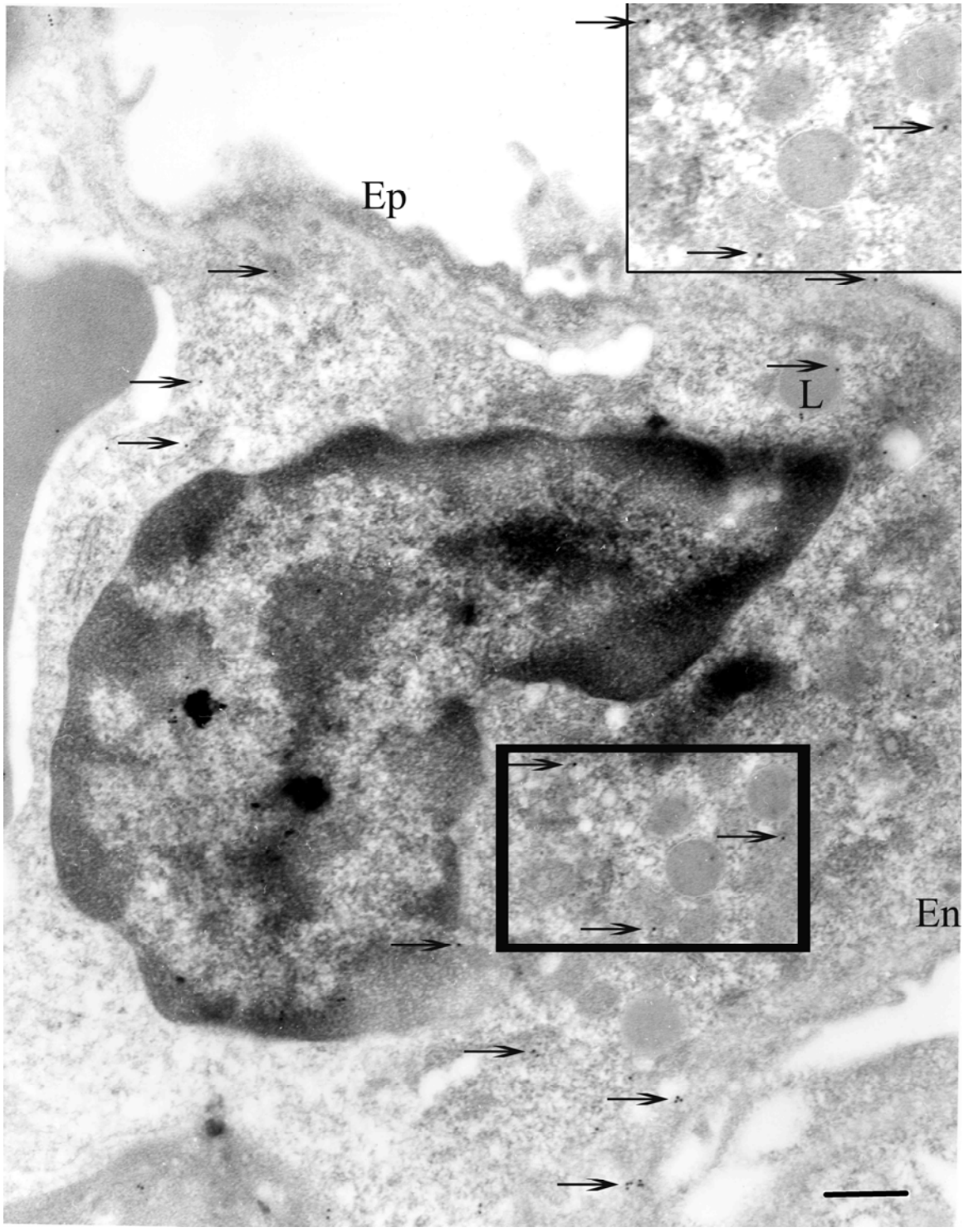

Figure 3.9. TGF-β2 immuno-electron microscopy.

A PIMM shows TGF-β2 staining (arrows and inset). En, endothelium; Ep, epithelium; L, lysosome. Scale bar=1 μm. Original magnification: 15,200X.

3.5. Discussion

This study provides data on the phenotype and possible functions of recruited PIMMs in lung inflammation in a rat model of sepsis. The data show recruitment of PIMMs following a single intraperitoneal challenge with *E. coli* and that recruited PIMMs react with a rat monocytes/macrophage antibody. The data further show increased expression of TNF-α, TGF-β2 and IL-10 in the lungs of rats treated with LPS following recruitment of PIMMs compared to those challenged with only LPS, as well as localization of these cytokines in PIMMs suggesting a proinflammatory role for these cells.

Animal models of sepsis show sequential recruitment of neutrophils and monocytes/macrophages into alveolar spaces (Laudes *et al.*, 2004; Andonegui *et al.*, 2003). There is considerable data on the recruitment and functions of neutrophils and monocytes into alveolar space (Mizgerd *et al.*, 2004; Johnston *et al.*, 2005; Maus *et al.*, 2002). Although it is accepted that monocytes/macrophages enter alveoli following a multi-step paradigm which includes vascular margination and effacements, very little attention has been paid to the recruitment and functions of intravascular monocytes/macrophages in lung inflammation. Previously, we have reported ultrastructural evidence that a single challenge with *E. coli* induces recruitment of PIMMs by 48 hours followed by their disappearance at 96 hours (Singh *et al.*, 1998). Now, we have used light and electron microscopic immunocytochemistry to show that recruited PIMMs in the rat react with ED-1 antibody. ED-1 antibody recognizes a lysosomal protein in rat monocytes/macrophages (Damoiseaux *et al.*, 1994; Dijkstra *et al.*, 1985). These data confirm that the cells recruited into the lung microvessels are monocytes/macrophages.

The functions of recruited PIMMs in lung inflammation remain largely unknown. We have previously shown that recruited PIMMs represent a transient population and their numbers peak at 48 hour in our model (Singh *et al.*, 1998). To determine their contributions to lung inflammation, we challenged rats with *E. coli* LPS at 48 hours post-*E. coli* infection and compared lung inflammation with normal rats injected with LPS. The histological data showed more intense lung inflammation characterized by vascular

congestion and recruitment of inflammatory cells following LPS challenge of PIMM-containing animals compared to the normal rats.

In an attempt to understand the mechanisms underlying histologic signs of increased lung inflammation in PIMM-containing LPS-challenged rats, we evaluated expression of TNF-α, IL-10 and TGF-β2. Immuno-electron microscopy localized TNF-α, IL-10 and TGF-β2 in recruited PIMMs in LPS-challenged rats. The use of ELISA showed that rats infected with *E. coli* and challenged with LPS showed higher lung concentrations of TNF-α, IL-10 and TGF-β2 compared to rats treated with LPS or the bacteria only. The localization of TNF-α in recruited PIMMs following LPS challenge is similar to the data from resident pulmonary intravascular macrophages (Singh *et al.*, 2004; Parbhakar *et al.*, 2005). Interestingly, while TNF-α induces vascular expression of adhesion molecules to promote recruitment of inflammatory cells in lung inflammation (O'dea *et al.*, 2005; Li *et al.*, 2004; Singh *et al.*, 2004), IL-10 and TGF-β are generally believed to be anti-inflammatory (Reidy and Wright, 2003; Ishii *et al.*, 1998; Cox, 1996; Cox *et al.*, 1995; Opal and DePalo, 2000a). However, recent data show that TGF-β produced by macrophages increases lung permeability while inhibition of TGF-β protected the endotoxin-challenged mice from lung edema as well as lung injury in hemorrhaged mice (Pittet *et al.*, 2001; Shenkar *et al.*, 1994). Our data show high levels of IL-10, which is an anti-inflammatory cytokine, in both control as well as those challenged with the bacteria and LPS. Interestingly, IL-10 levels were suppressed in rats that received only LPS. IL-10 can inhibit the release of proinflammatory cytokines such as TNF-α and IL-1β from monocytes and macrophages to prevent overexpression of inflammation and to stimulate resolution of lung inflammation (Kasama *et al.*, 1994; Fiorentino *et al.*, 1991; Ishida *et al.*, 1992; Cox, 1996; Lo *et al.*, 1998). The increased expression of IL-10 along with that of TNF-α is intriguing but not surprising because the outcome of an inflammatory episode is determined not by a single cytokine but through a complex interplay of myriad of inflammatory mediators. For example, lower levels of IL-10 and higher levels of TNF-α in peripheral blood and bronchoalveolar lavage of sepsis/AIDS patients can predict more inflammatory disease (Armstrong and Millar, 1997). Therefore, the competing expression of pro- and anti-inflammatory cytokines such as TNF-α and IL-10, respectively, may determine severity of inflammation, tissue

damage and mortality. Our data showing increased expression of IL-10, TNF-α and TGF-β2 in conjunction with lung inflammation but no mortality in PIMM-containing LPS-challenged rats compared to control rats administered LPS may suggest tilting of balance in favor of TNF-α. These observations suggest that PIMMs in LPS-challenged animals could contain and contribute multiple cytokines to the complex inflammatory process in the lung.

We conclude that PIMMs are recruited in inflamed lungs and that recruited PIMMs contain multiple cytokines. Similar to resident PIMMs, the recruited PIMMs appear to play a role in modulating lung inflammation in response to a secondary challenge. We are aware that our data were collected at only one time point following the treatments and that lung inflammation is a dynamic process. Furthermore, the localization of multiple cytokines in recruited PIMMs creates a need to delineate precise functions of each of these mediators.

CHAPTER 4: MULTIPLE EXPOSURES TO SWINE BARN AIR INDUCE LUNG INFLAMMATION AND AIRWAY HYPERRESPONSIVENESS*

4.1. Abstract

Swine farmers repeatedly exposed to the barn air suffer from respiratory diseases. However the mechanisms of lung dysfunction following repeated exposures to the barn air are still largely unknown. Therefore, we tested a hypothesis in a rat model that multiple interrupted exposures to the barn air will cause chronic lung inflammation and decline in lung function. Rats were exposed either to swine barn (8 hours/day for either one or five or 20 days) or ambient air. After the exposure periods, airway hyper-responsiveness (AHR) to methacholine (Mch) was measured and rats were euthanized to collect bronchoalveolar lavage fluid (BALF), blood and lung tissues. Barn air was sampled to determine endotoxin levels and microbial load. The air in the barn used in this study had a very high concentration of endotoxin (15361.75 ± 7712.16 EU/m^3). Rats exposed to barn air for one and five days showed increase in AHR compared to the 20-day exposed and controls. Lungs from the exposed groups were inflamed as indicated by recruitment of neutrophils in all three exposed groups and eosinophils and an increase in numbers of airway epithelial goblet cells in 5- and 20-day exposure groups. Rats exposed to the barn air for one day or 20 days had more total leukocytes in the BALF and 20-day exposed rats had more airway epithelial goblet cells compared to the controls and those subjected to 1 and 5 exposures ($P<0.05$). Bronchus-associated lymphoid tissue (BALT) in the lungs of rats exposed for 20 days contained germinal centers and mitotic cells suggesting activation. There were no differences in the airway smooth muscle cell volume or septal macrophage recruitment among the groups. We conclude that multiple exposures to endotoxin-containing swine barn air induce AHR, increase in mucus-containing airway epithelial cells and lung inflammation. The data also show that prolonged multiple exposures may also induce adaptation in AHR response in the exposed subjects. * Respiratory Research (2005) **6**: 50. http://respiratory-research.com/content/6/1/50

4.2. Introduction

Respiratory diseases in agricultural workers are one of the earliest recognized occupational hazards (Schenker *et al.*, 1998). Swine farmers work in confined buildings in close proximity to a large number of pigs and are exposed to toxic gasses such as ammonia and hydrogen sulfide, and to high levels of dust and endotoxins (Asmar *et al.*, 2001). Exposure to such toxic bio aerosols including endotoxins in the barn air is a risk factor for the development of chronic respiratory symptoms and lung dysfunction (Zejda *et al.*, 1994; Zejda *et al.*, 1993; Frevert and Warner, 1999). Workers exposed to barn air report significantly higher frequencies of respiratory symptoms, cold, chest illness and pneumonia (Asmar *et al.*, 2001; Zejda *et al.*, 1994). The severity of lung irritation and respiratory symptoms increases during winter and is also related to the number of working hours (Iversen *et al.*, 2000). Single, 3-5 hour exposure of naïve, healthy, non-smoking subjects to swine barn air increases IL-6 in serum and IL-6 and IL-8 in nasal lavage and inflammatory cells in bronchoalveolar lavage fluid (BALF) (Larsson *et al.*, 1994; Dosman *et al.*, 2000). Furthermore, pig barn dust stimulates IL-8 and IL-6 release from human bronchial epithelial cells *in vitro* (Romberger *et al.*, 2002). Collectively, these data show that a single exposure to the barn air initiates acute lung inflammation.

Although swine barn workers are repeatedly exposed to barn air, majority of studies have focused on the acute pulmonary effects of single exposure (Larsson *et al.*, 1994; Palmberg *et al.*, 2004). Multiple exposures to barn air are linked to chronic lung inflammation including chronic bronchitis, decline in lung function and higher incidence of asthma (Zejda *et al.*, 1994; Palmberg *et al.*, 2002; Pedersen *et al.*, 1996). Pig farmers with an average exposure history of 10.5 years and a daily exposure of 6.6 hours show significantly lower forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) compared to unexposed control subjects (Zejda *et al.*, 1994). Interestingly, acutely exposed naïve volunteers, show significantly more lung dysfunction, AHR, increase in cytokine levels and inflammatory cell numbers in blood and nasal lavage compared to the pig barn workers repeatedly exposed to the barn air

(Larsson *et al.*, 1994; Wang *et al.*, 1997; Palmberg *et al.*, 2002). These data suggest induction of an adaptive response in subjects repeatedly exposed to the barn air.

There is paucity of data on *in situ* cellular and molecular changes following multiple exposures to pig barn air. This is largely because of lack of an animal model to investigate the physiological impact of exposure to barn air. Therefore, we decided to undertake an *in vivo* single and multiple exposure study using rats to characterize cellular and molecular responses. We hypothesized that single and multiple exposures to swine barn air will induce lung inflammation and a decline in lung function. The data show that single and multiple exposures cause increase in AHR, inflammatory cells in BALF, mucus cells in the airways and lung inflammation.

4.3. Materials and Methods

4.3.1. Rats and treatment groups

The experimental protocols were approved by the University of Saskatchewan Campus Committee on Animal Care and experiments were conducted according to the Canadian Council on Animal Care Guidelines. Specific pathogen-free, six-week-old, male, Sprague-Dawley rats (Charles River Laboratories, Canada) were maintained in the animal care unit of Western College of Veterinary Medicine. Rats were randomly divided into four groups (n=6 each). All personnel involved in collection and analyses of samples were blinded to the treatment groups.

4.3.2. Exposure to swine barn air

We selected a regular commercial swine barn in the village of Aberdeen in Saskatchewan. The barn chosen for study had 60 dry sows and three boars. These pigs were fed with ground barley. Rat cages were hung from the barn ceiling at an approximate height of two meters above the floor. Groups of rats were exposed to barn air either for eight-hours for one-day, 5 days or for four cycles of 5 days (8 hours/day) each followed by 2 days in normal ambient air after every cycle. When rats were not exposed to the barn air, they were kept with the control animals in normal ambient air. Control rats were treated similarly except that they were not exposed to the barn air.

4.3.3. Barn air sampling for endotoxin analysis

We sampled the barn air twice weekly to determine endotoxin levels as described previously (Kirychuk *et al.*, 1998). Briefly, we collected airborne barn dust onto a pre-weighted, binder-free glass fibre inline filter (SKC Edmonton, Canada) hung at the level of rat cages. Barn air was drawn through the sampler (DuPont Air Sampler) for eight hours on each sampling day. The average flow-rate of the sampler was noted before and after each sampling period. Filters were desiccated before and after sampling. After weighing, the filters were placed in 50-mL polypropylene centrifuge tubes and were stored at 4°C until endotoxin analysis.

Endotoxin analysis was performed as described elsewhere (Kirychuk *et al.*, 1998). Briefly, the filters with collected dust were washed individually in centrifuge tubes with 10 mL of sterile pyrogen-free water (DIN 00624721; Astra Pharma Inc; Mississauga, ON, Canada) followed by incubation for one-hour at room temperature in a sonicating water bath. Serial two-fold dilutions of the supernatant fluids were analyzed for Gram-negative bacterial endotoxin using an end-point assay kit as recommended by the manufacturer (model QCL-1000; Cambrex Bioscience Inc.; Walkersville, MD). The endotoxin standard (*Escherichia coli* O111:B4) was used in duplicate at four concentrations (0.1 to 1.0 endotoxin units (EU)/mL) in each assay to generate the standard curve. The lower detection limit was 0.1 EU/mL, which is equivalent to 1.0 EU per filter. The sampling time and flow rate were used to calculate the concentration of endotoxin in air (EU/m^3).

4.3.4. Viable microbial count

Viable microbial count was achieved using a six-stage viable cascade impactor (Graseby, Smyrna, GA). Air samples were collected from the vicinity of the rat cages hung from the ceiling of the barn by using a vacuum pump that was attached to the impactor capable of drawing air through the impactor at a rate of 1 ft^3/ min (28.3 L/min). Six media plates of Tryptic Soy Agar with 5% sheep's blood were placed in the sampler and airborne microbes were directly collected onto 20 mL of media in 100 mm petri dishes. The air was drawn through the impactor for a duration of 15 seconds. The procedure was performed twice every week. The cascade impactor was cleaned

thoroughly with 70% ethanol between each collection event. The plates were incubated at 37 ^{o}C for 18-24 hours, and the colonies were counted using the positive-hole method correcting for microbial coincidence (ANDERSEN, 1958). Bacterial colonies associated with stage-6 (0.65-1.1 μm) to stage-2 (4.7-7 μm) were classified as, 'respirable" and those associated with stage-1 (≥ 7 μm) as, 'non-respirable'.

4.3.5. Measurement of airway hyper-responsiveness

AHR was measured within 2-3 hours after completion of barn or ambient air exposures in awake control and barn exposed-rats in response to increasing concentrations of methacholine (Mch) using head-out whole body plethysmography (Neuhaus-Steinmetz *et al.*, 2000). Air was supplied to the head and body compartments of the plethysmograph through a small animal ventilator (Kent Scientific, Litchfield, CT) and changes in respiratory airflow were monitored using a flow sensor (TRS3300; Kent Scientific, Litchfield, CT) linked via a preamplifier and A/D board (Kent Scientific) to a computer-driven real-time data acquisition/analysis system (DasyLab 5.5; DasyTec USA, Amherst, NH). The compartment of the plethysmograph, which accommodates the animal's head, was connected to an ultrasonic nebuliser (UltraNeb 99; Devilbiss Co., Somerset, PA) to expose the rats to Mch (Sigma Chemical Co. St. Louis, MO) (Vijayaraghavan *et al.*, 1993; Vijayaraghavan *et al.*, 1994). Each rat was sequentially exposed to aerosols of saline alone (Mch 0 mg/ml) and then increasing doses of Mch diluted in saline (0.75, 1.5 and 3.0 mg/mL) and Flow@50%Tve1 (lung airflow at 50% of the expiratory tidal volume) was noted for saline and each of the Mch concentrations.

4.3.6. Blood, bronchoalveolar lavage, tissue collection and processing

At the end of the exposure period, rats were euthanized (1 mg xylazine and 10 mg ketamine / 100g) and blood, BALF and lung samples were collected. Blood was collected by cardiac puncture for differential and total leukocyte counts. BALF was collected by washing the whole lung with 3 ml of ice cold Hanks Balanced Salt Solution (Sigma Chemicals Co., St. Louis, MO). Three pieces from each lung lobe

(left and right) were fixed in 4% paraformaldehyde for 16 hours and embedded in paraffin for light microscopy. Haematoxylin and eosin stained sections were used for histopathological evaluation of pulmonary inflammation.

4.3.7. Quantification of mucus-producing cells

Mucus-producing goblet cells were quantified in lung sections stained with Periodic-acid Schiff (PAS) reagent (Leigh *et al.*, 2004). Images were captured with the 20X objective lens of an Olympus microscope (Olympus BH2) connected to a digital camera (DVC Digital Camera, Diagnostic Video Camera Company, Austin, TX 78736-7735). The images were analysed using image analyses software (Northern Eclipse, version 6; Empix Imaging Inc., Mississauga, ON, Canada). Only those bronchi with a length to width ratio of less than 2.5 were selected for counting PAS-positive cells so as to minimize the error that might arise from tangential sectioning (Brass *et al.*, 2003). The PAS-positive goblet cells were counted manually and normalized to the length of the bronchial epithelial perimeter on the basal side, and expressed as the number of PAS-positive cells per mm of basement membrane.

4.3.8. Immunohistochemistry

Lung sections were processed for immunohistochemistry as described previously (Singh *et al.*, 2001). Briefly, the sections were deparaffinized, hydrated and incubated with 5% hydrogen peroxide for 30 minutes to quench endogenous peroxidase, treated with pepsin (2mg/ml in 0.01N HCl) for 45 minutes to unmask the antigens and blocked with 1% bovine serum albumin for 30 minutes. Sections were incubated with primary antibodies against rat macrophage (1:400; ED-1, Serotec Inc**.** NC, USA) or monoclonal mouse anti-human smooth muscle actin (1:50; clone 1A4; DAKO A/S, Denmark), followed by appropriate biotinylated or horseradish peroxidase (HRP) -conjugated secondary antibodies (1:150; DAKO A/S, Denmark). Sections incubated with biotinylated antibodies were incubated with HRP conjugated streptavidin (1:300, DAKO A/S, Denmark) before color development. The reaction was visualized using a color development kit (VECTOR -VIP, Vector laboratories, USA). Controls consisted of

staining without primary antibody or with isotype matched immunoglobulin instead of primary antibody.

4.3.9. Quantification of macrophages and airway smooth muscle

ED-1 positive macrophages in the septa were counted in 20-high power fields (using 40X objective covering an area of 9.6 mm^2). For smooth muscle quantification, a method described by Leigh *et al.* (Leigh *et al.*, 2002) was followed with a slight modification. A line was drawn along the outer border of the positively stained smooth muscle area and total stained area within that circle was measured using Northern Eclipse image analyses software. Next, a similar line was drawn along the inner border of the airway smooth muscle area to demarcate and measure the stained area. Stained area within the line drawn along smooth muscle inner border was deducted from the stained area within line drawn along smooth muscle outer border, to obtain the total stained area of airway smooth muscle. This total stained area of airway smooth muscle was normalized to the length of the outer perimeter of the airway smooth muscle, and results were expressed as, smooth muscle stained area in mm^2 per mm of airway smooth muscle perimeter.

4.3.10. Statistical analyses

All data were expressed as mean ± SD. Group differences were examined for significance using one-way analysis of variance or two-way repeated measures analysis of variance with Fishers LSD as *post hoc* test (Sigma Stat Version 2.0, SPSS Inc., Chicago, IL 60611). Significance was established at $P<0.05$.

4.4. Results

4.4.1. Barn air characterization

The mean endotoxin concentration in the swine barn air for the period of exposure was 15361.75± 7712.16 EU/m^3 of air. The amount of endotoxin in air samples from the room where control animals were kept (normal ambient air) was below the level of detection. The levels of endotoxin in the barn air in our study are much higher than those reported by other researchers (Senthilselvan *et al.*, 1997b; Zejda *et al.*, 1994). The total

viable aerobic bacterial counts in the barn air during the exposure period are shown in Table 4.1. Air samples collected from the room where control rats were kept did not yield any bacterial colonies.

4.4.2. Airway hyper-responsiveness (AHR)

Inhalation of increasing concentrations of Mch caused decrease in airflow (Flow@50%Tve1) indicating airway reactivity and broncho-constriction. The data showed group differences in percent decrease in Flow@50%Tve1 (Figure 4.1; $P<0.001$). Both 1- and 5-day exposed rats showed increased AHR compared to controls ($P<0.001$) and 20-day exposed ($P<0.05$). However, there were no differences in AHR between the control and 20-day exposed ($P=0.207$) and 5-day and 1-day ($P=0.249$) exposed rats.

4.4.3. BALF cell counts

There were differences in total leukocyte counts in BALF among the four groups (Figure 4.2A; $P<0.001$). The one day exposure group had higher BALF total leukocytes compared to the control, 5-day or 20-day exposed rats ($P<0.001$). The 20-day exposed animals contained higher numbers of total leukocytes than control ($P=0.01$) and those exposed for 5 days ($P=0.008$). BALF total leukocytes were not different between control and 5-day exposed rats ($P=0.932$).

The increased BALF total leukocytes in single exposure group, compared to control, 5- and 20-day exposed rats, were characterised by increased absolute neutrophil, macrophage and lymphocyte numbers (Figure 4.2B-D; $P<0.001$). Increased BALF total leukocytes in 20-day exposed rats were characterized by increased absolute neutrophil (from controls, $P=0.022$) and macrophage (control and 5-day exposed rats, $P<0.001$) numbers. BALF absolute eosinophil numbers did not differ among the four groups ($P=0.178$).

4.4.4. Blood cell counts

There was no difference among the groups for total leukocyte counts (Figure 4.3A; $P=0.090$). However, the absolute neutrophil numbers were different among the four groups (Figure 4.3B; $P<0.001$). Rats exposed for 20 days showed higher absolute

neutrophil numbers compared to the control and those exposed for 1 or 5 days (P<0.001). Furthermore, rats exposed for 1 day showed higher blood absolute neutrophils when compared to 5-day exposed rats (P=0.038). Blood absolute monocyte numbers did not differ among the four groups (Figure 4.3C; P=0.122). Blood absolute lymphocyte numbers were different among the four groups (Figure 4.4D; P<0.001). Compared to 20-day exposed, control (P=0.003), 1-day (P<0.001) and 5-day (P=0.011) exposed rats showed increased numbers of blood absolute lymphocytes.

4.4.5. Histopathology

Lung sections from control rats showed normal histology (Figure 4.4.A) while those exposed for 1 day, 5 (Figure 4.4.B-C) or 20 days (not shown) showed neutrophil infiltration into the lung tissue. Lung sections from 5-day (Figure 4.4.D) and 20-day (not shown) exposed rats manifested perivascular and peribronchial eosinophil infiltration. Bronchus-associated lymphoid tissue (BALT) showed germinal centres and mitotic cells indicating BALT activation in rats exposed for 20 days (Figure 4.4.F) compared to the controls (Figure 4.4.E) or those subjected to 1 and 5 exposures (data not shown).

4.4.6. Mucus cell quantification

Because PAS method stains mucus as pink, it is commonly used as a method to identify mucus-containing cells (Figure 4.5). Morphometric data revealed more PAS-positive mucus-containing goblet cells in the airways of rats exposed for 5 or 20 days compared to the controls (5-day: P=0.040; 20-day: P<0.001) and 1-day (5-day: P=0.007; 20-day: P<0.001) exposed rats (Figure 4.5A-D). Furthermore, rats exposed 20 times contained more airway mucus cells compared to the 5-day exposure group (P<0.001). There was no difference between control and 1-day exposed rats (P=0.435).

4.4.7. Quantification of ED-1 positive macrophages

The numbers of macrophages in the alveolar septa, stained with ED-1 antibody were not different among the four groups (Figure 4.6; P=0.350).

4.4.8. Immunohistochemical quantification for smooth muscle actin (SMA)

We used anti-human SMA antibody, which cross reacts with rat tissue to stain smooth muscles around the bronchi, bronchioles and blood vessels. Morphometric analyses showed no differences in smooth muscle area among the groups (Figure 4.7; P=0.681).

Table 4.1. The total, respirable and non-respirable aerobic viable bacterial count (CFU/m^3 of air sampled) from the barn air.

Classification	Viable aerobic bacterial count X 10^4 (CFU/m^3 of sampled air)*
Total	12.10 ± 8.47
Respirable (0.65-7 μm)	4.85 ± 4.97
Non-respirable (≥ 7 μm)	7.26 ± 7.50

* Viable bacterial counts are expressed as Mean ± SD.

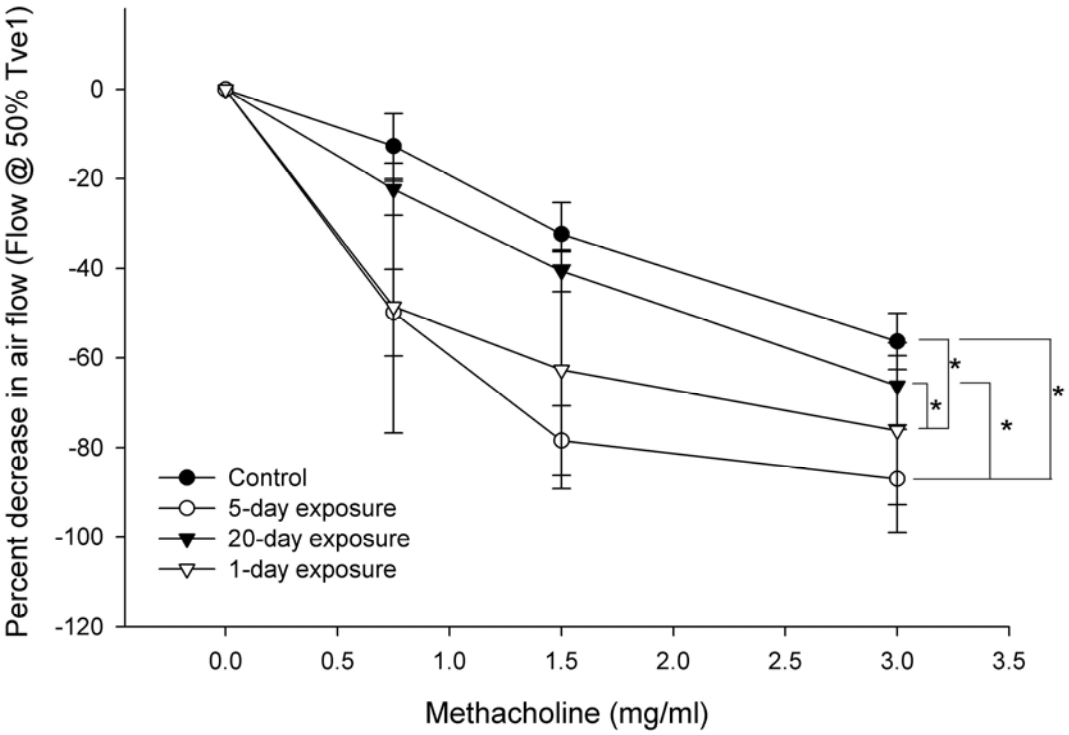

Figure 4.1. Airway hyper-responsiveness.

Airway hyperresponsiveness to methacholine challenge in rats was measured using a whole-body head-out plethysmograph. Compared to controls, both 1-day and 5-day ($P<0.001$) exposed rats showed increased airway hyperresponsiveness. Compared to 20-day exposed rats, 5-day ($P=0.001$) and 1-day ($P=0.014$) exposed rats showed increased airway hyper-responsiveness. There was no difference between control and 20-day exposed ($P=0.207$) and 1-day and 5-day exposed ($P=0.249$) rats. *: Significantly different from other groups as indicated by line/s.

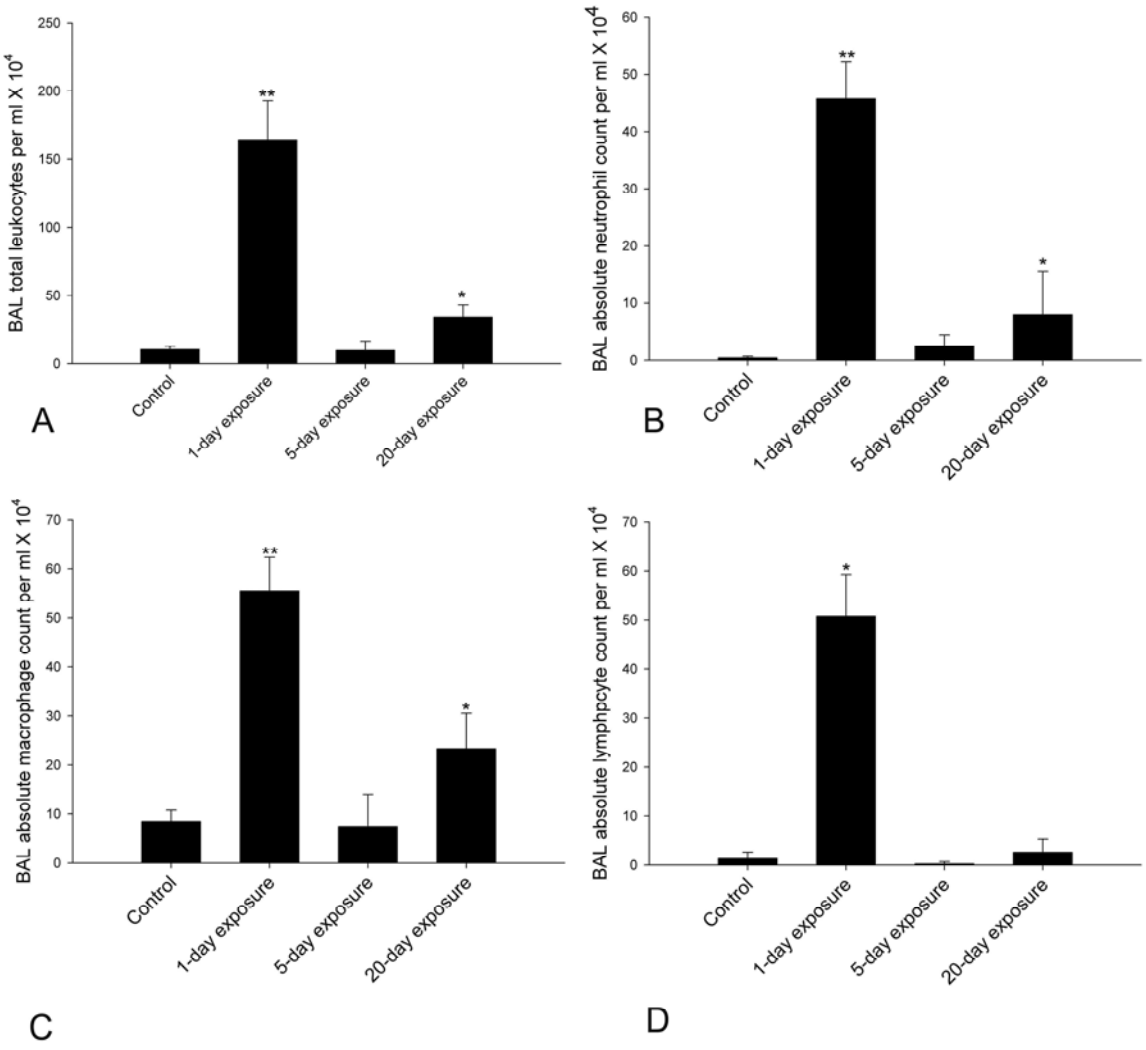

Figure 4.2. Total and differential leukocytes in the bronchoalveolar lavage fluid.

Bronchoalveolar lavage was performed on the whole lung using 3 ml of cold HBSS. Cells were counted using a hemocytometer. Cytospins were prepared from BAL fluid and cells were differentiated with Wright's staining.

4.2A. BALF total leukocyte counts.
BALF total leukocytes were different among the four groups ($P<0.001$). Compared to controls, 5-day and 20-day exposed, 1-day exposed rats showed increased numbers of BALF total leukocytes ($P < 0.001$). Rats exposed for 20 days showed increased numbers of BALF total leukocytes when compared to controls ($P=0.01$) and 5-day ($P=0.008$) exposed rats. 5-day exposed rats did not differ from controls in their BALF total leukocyte numbers ($P=0.932$). ** Significantly different from control, 5-day and 20–day exposed rats and * significantly different from control, 1-day and 5-day exposed rats.

4.2B. BALF absolute neutrophil counts.
BALF absolute neutrophil counts were different among the groups ($P<0.001$).1-day exposed rats showed higher BALF absolute neutrophils when compared to control, 5-day and 20-day exposed rats ($P<0.001$). 20-day exposed rats showed higher BALF absolute neutrophil count when compared to control rats ($P=0.022$). There was no difference between control and 5-day exposed ($P=0.538$) and 20-day and 5-day exposed ($P=0.119$) rats. ** Significantly different from control, 5-day and 20-day exposed rats and * significantly different from control.

4.2C. BALF absolute macrophage counts.
BALF absolute macrophage count was different among the four groups ($P<0.001$). BALF absolute macrophage count was higher in 1-day exposed when compared to control, 5-day and 20-day exposed rats ($P<0.001$). 20-day exposed rats showed higher BALF absolute macrophage count when compared to control and 5-day ($P<0.001$) exposed rats. There was no difference between control and 5-day exposed rats ($P=0.789$). ** Significantly different from control, 5-day and 20-day exposed rats and * indicates significantly different from control and 1-day and 5-day exposed rats.

4.2D. BALF absolute lymphocyte count.
BALF absolute lymphocyte count was different among the four groups ($P<0.001$). BALF absolute lymphocyte count was higher in 1-day exposed when compared to control, 5-day and 20-day exposed rats ($P<0.001$). * Significantly different from other three groups.

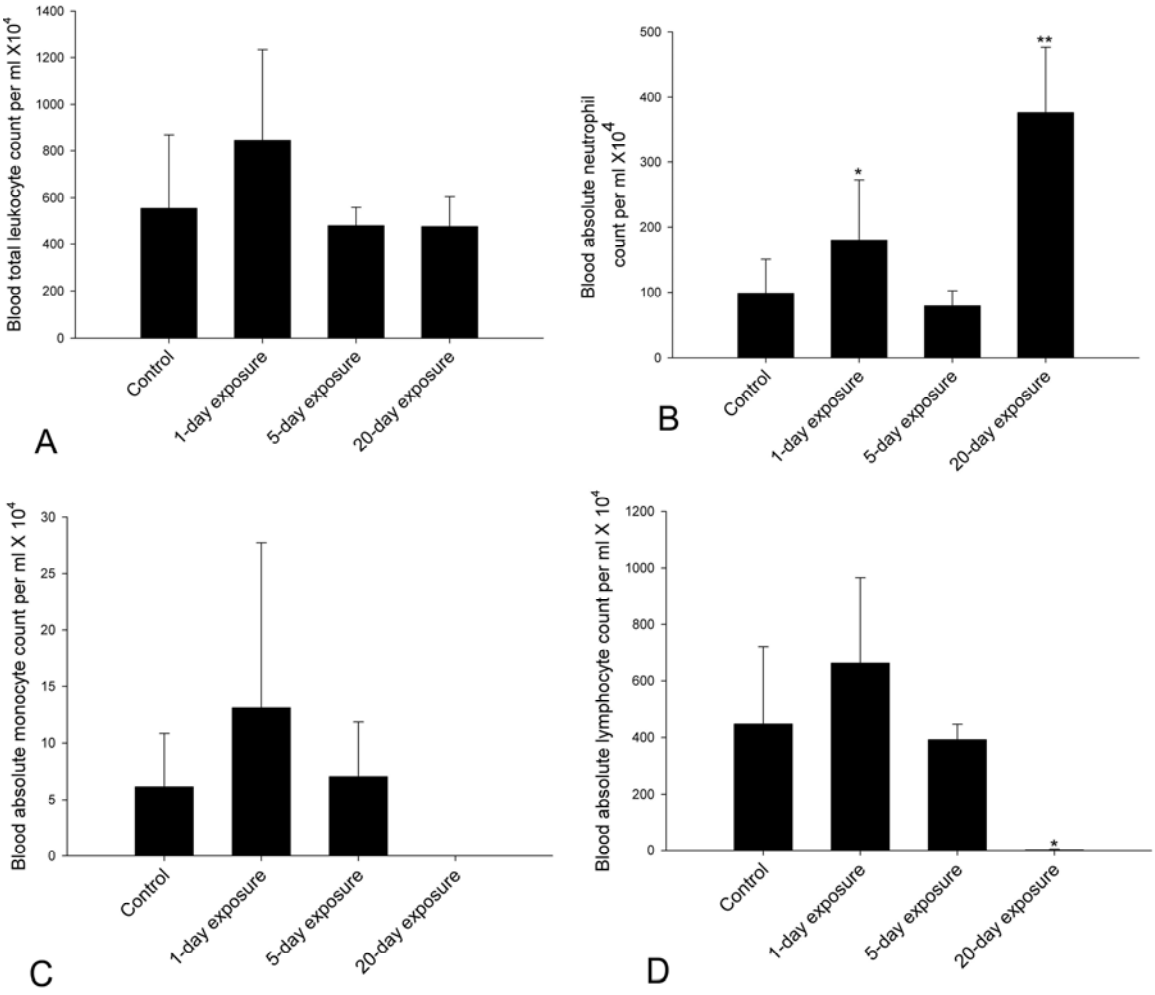

Figure 4.3. Total and differential leukocyte count in blood

Blood total leukocytes were counted using hemocytometer and smears were differentiated with Wright's stain. 4.3A. Blood total leukocyte count did not differ among the groups (Figure 4.3A; P=0.090). 4.3B. Blood absolute neutrophil count was different among the four groups (Figure 4.3B; P<0.001). 20-day exposed rats showed higher blood absolute neutrophils count when compared to control, 1-day and 5-day exposed rats (P<0.001). 1-day exposed rats showed higher blood absolute neutrophil count when compared to 5-day exposed rats (P<0.038). Both 1-day (P=0.073) and 5-day exposed rats (P=0.678) did not differ from controls. ** Indicate significantly different from control, 1-day and 5-day exposed rats and * indicate significantly different from 20-day and 5-day exposed rats. 4.3C. Blood absolute monocyte count did not differ among the four groups (Figure 4.3C; P=0.122). 4.3D. Blood absolute lymphocyte count was different among the four groups (Figure 4.3D; P<0.001).Compared to 20-day exposed, control (P=0.003), 1-day (P<0.001) and 5-day (P=0.011) exposed rats showed increased numbers of blood absolute lymphocytes. * indicates significantly different from other three groups.

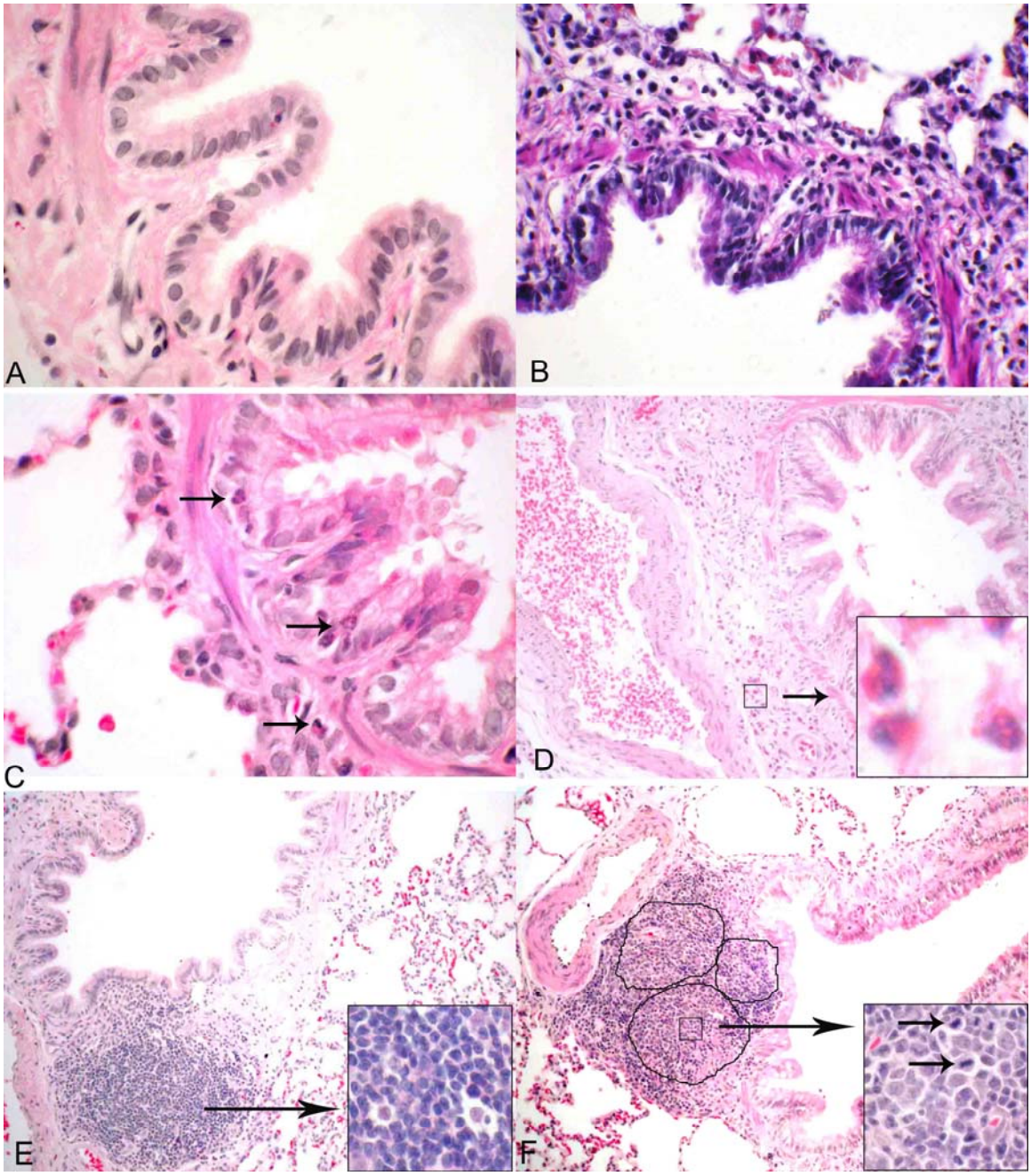

Figure 4.4. Histopahtological evaluation of lung sections.

Histopathological changes in the lungs of swine barn air exposed and control rats were evaluated using hematoxylin and eosin stained sections. Control rat lungs (A) showed no inflammatory cell infiltration. Among the exposed groups, 1-day (B), 5-day (C) and 20-day exposed rats (not shown) showed peribronchiolar neutrophilic (C; arrows) and 5-day (D) and 20-day exposed (not shown) showed eosinophilic (D; arrows and inset) infiltration. Bronchus-associated lymphoid tissue (BALT) in control (E), 1-day and 5-day exposed (both not shown) appeared normal and had no germinal centers, whereas 20-day exposed rat lungs had activated BALT with germinal centers (F; outlined in black line)

containing several mitotic cells (F; inset). Original magnification A- C: X400; D-F: X100; Insets: X1000

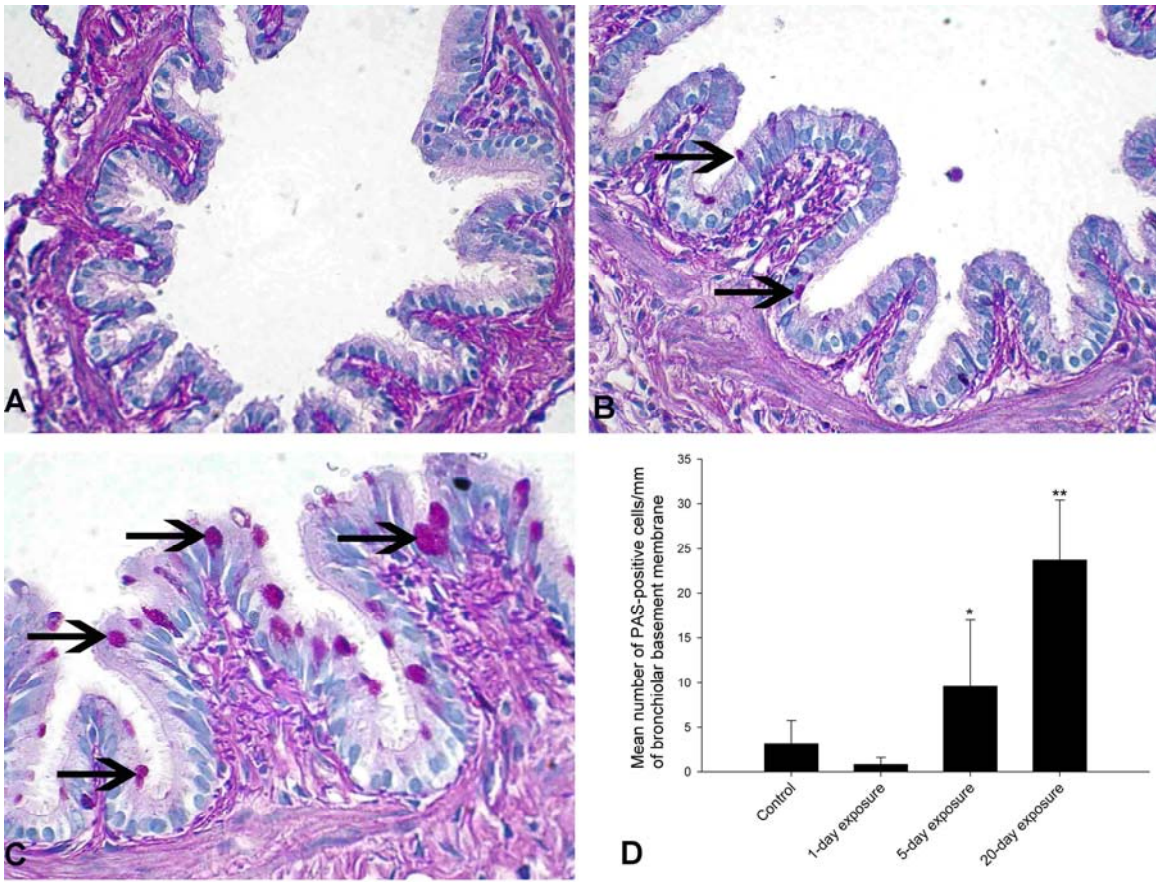

Figure 4.5. Quantification of mucus producing cells in the airways.

Mucus producing goblet cells in the airways were quantified using PAS staining. Control rats showed no mucus producing cells in the bronchioles (A). 5-day exposed and 20-day exposed rats showed large number of mucus producing cells (B&C; arrows). Quantification of PAS-positive cells showed a significantly higher number of cells in 5-day and 20-day exposed rat lungs compared to the controls (5-day: $P=0.040$; 20-day: $P<0.001$) and one-day (5-day: $P=0.007$; 20-day: $P<0.001$) exposed rats (Figure D). Also, the increase in mucus producing cells was higher in 20-day exposed compared to 5-day exposed rat lungs ($P<0.001$). Number of mucus producing cells did not differ between control and 1-day exposed rats ($P=0.435$). *: Significantly different from control, 1-day and 20-day exposure. **: Significantly different from control, 1-day and 5-day exposure. The bars represent mean ± SD. Original magnification A-C; X400

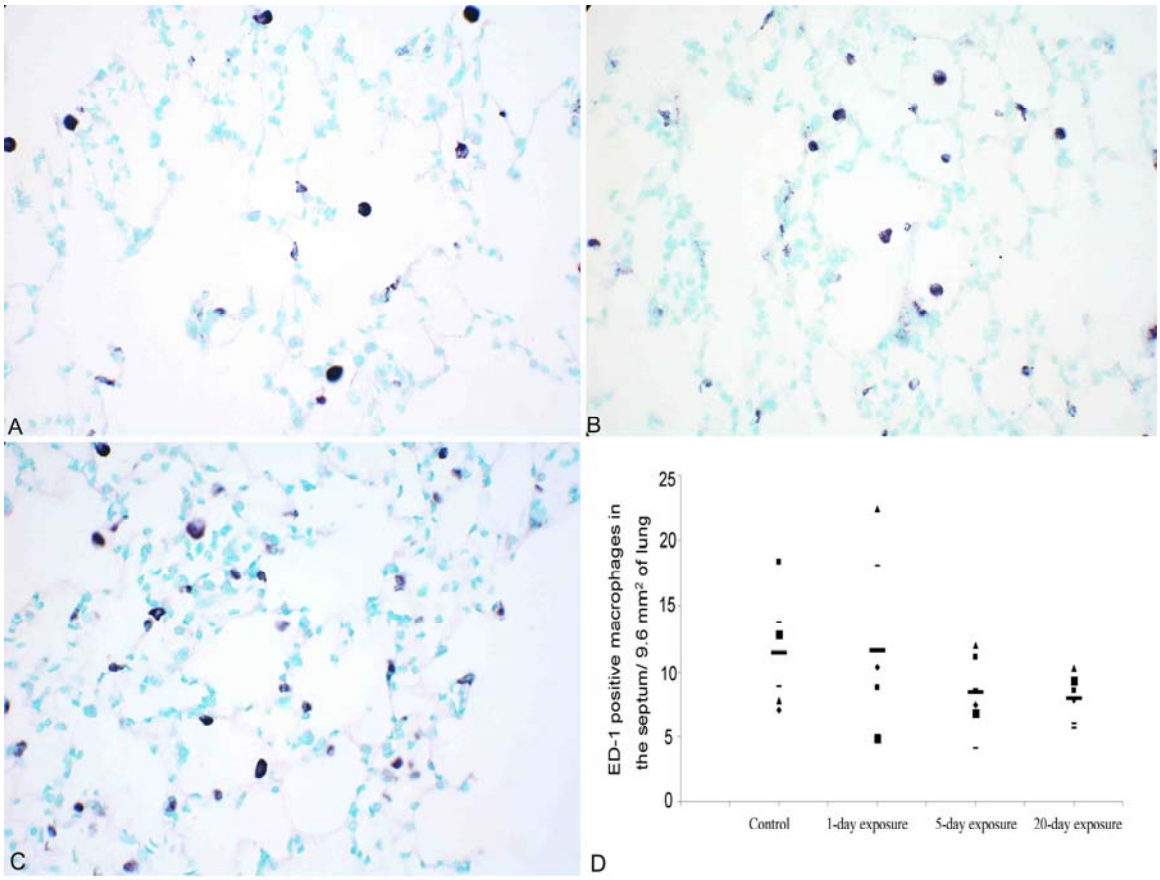

Figure 4.6. Quantification of septal macrophages in the lung.

Macrophages were stained using ED-1 antibody. Lungs from control (A), 1-day (not shown in picture), 5-day exposed (B) and 20-day exposed (C) rats appeared to have similar numbers of septal macrophages. To confirm this we quantified ED-1 positive cells in the septum. D: Is a scatter plot showing number of ED-1 cells in the septum, in different groups. The horizontal bars in each group represent the mean for that particular group. There was no difference between the groups (P=0.350). Original magnification A-C: X400

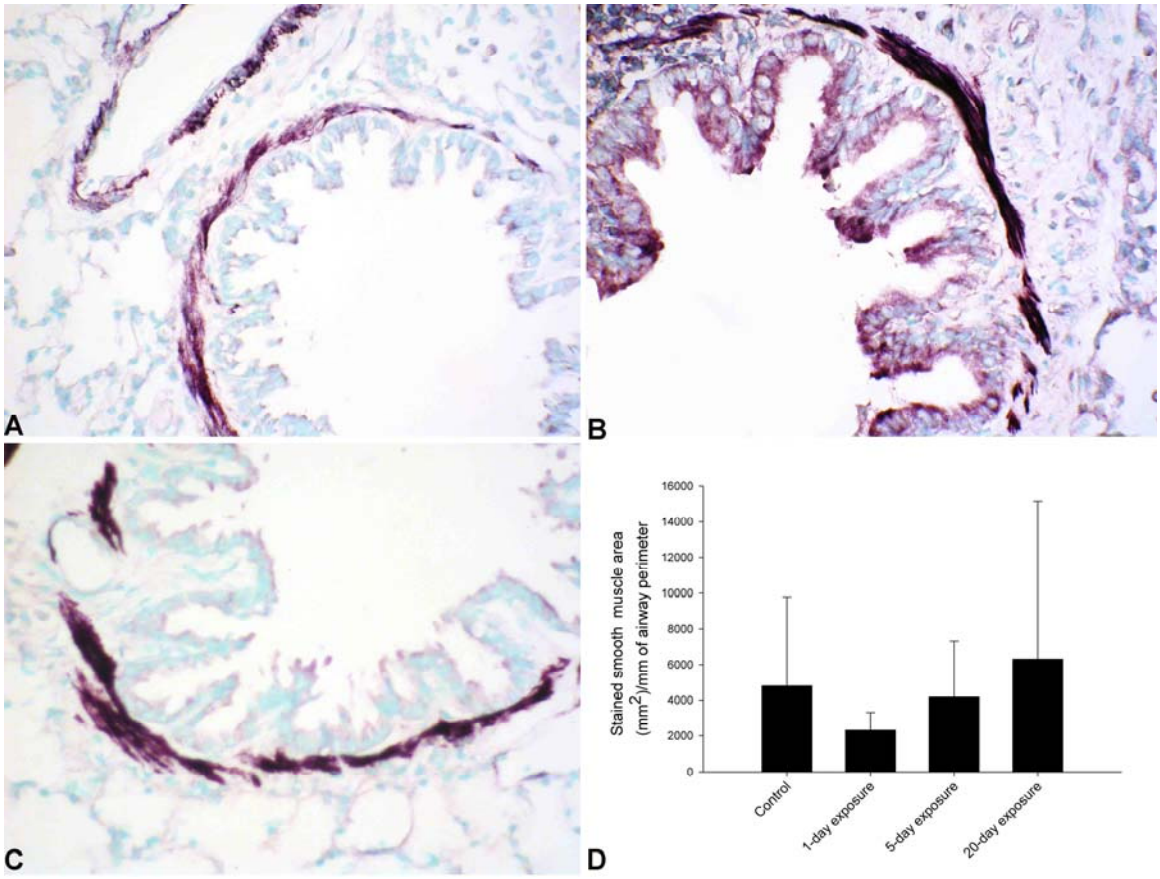

Figure 4.7. Airway smooth muscle quantification.

The staining pattern for smooth muscle in controls (A), 1-day (not shown in picture), 5-day exposed (B) and 20-day exposed (C) rat lungs appeared similar. D. The stained area of smooth muscle around the bronchioles was measured using image analyses software. The area of smooth muscle was not significantly different between the groups (P=0.681). Original magnification: A-C; X400

4.5. Discussion

We report *in vivo* and *in situ* data using an animal model on the effects of single and multiple exposures to the swine barn air. The data show that exposures to swine barn air induce an initial increase in AHR in one and five day exposed rats followed by an adaptive response in 20-day exposed rats; the 20-day group resembled the controls. Swine barn exposure induced lung inflammation in all the exposed groups characterized by infiltration of inflammatory cells, activation of BALT in 20-day exposed rats and an increase in mucus cells in the airway epithelium of 5- and 20-day exposed rats.

Our data show that one and five exposures to barn air induce significantly greater AHR in rats compared to 20 exposures and the unexposed. The AHR observed after 20 exposures was not different from controls. The precise mechanisms of increased AHR following one or five exposures to the barn air and an apparent adaptive response after 20 exposures remain incompletely understood. Previously, it was speculated that similar airway responses in the barn workers are initiated by the endotoxin present in the barn air (Larsson *et al.*, 1994; Cormier *et al.*, 1997). It is likely that high levels of endotoxin in the barn air observed in our study are partially contributing to lung dysfunction induced in the exposed rats. Endotoxin in house dust has also been identified as a cause of lung dysfunction, which is characterized by increased AHR and inflammation (McKinley *et al.*, 2004). Notwithstanding the cause of AHR following exposure to the highly complex barn air, there was amelioration of AHR in rats exposed for 20 days in conjunction with persistent inflammation. Previous data from a mouse model of allergic and IL-6 induced lung inflammation have shown dissociation between intensity of AHR and the lung inflammation (DiCosmo *et al.*, 1994; Kobayashi *et al.*, 2003). Thus, our observations show that multiple exposures to barn air, which contains many toxic aerosols including endotoxins and ammonia, initially show an increase in AHR followed by an adaptive response. These data from exposed rats parallel the observations from barn workers who showed initial increase in AHR and decreased FEV1, FVC and mid-expiratory flow (FEV 25-75) followed by an adaptation indicated by less severe AHR (Senthilselvan *et al.*, 1997a; Bessette *et al.*, 1993). Based on the similarity in lung responses following

exposure to the barn air, the rat may be a good model to investigate *in vivo and in situ* cellular and molecular aspects of lung dysfunction in pig barn workers.

Rats, following single and 20 exposures, demonstrated more neutrophils and macrophages in their BALF. Rats exposed 20 times showed activation of BALT compared to the control and those exposed for 1 or 5 times indicating a progression towards chronic inflammation. BALT activation similar to that observed in our study has been reported in chronic bacterial infection (Rodriguez *et al.*, 2004; Iwata and Sato, 1990), and following exposure to endotoxin and diesel exhaust (Ermert *et al.*, 2002; Ermert *et al.*, 2000). Lung sections from all the exposed groups contained perivascular and peribronchial infiltration of inflammatory cells. It is well established that inflammatory cells are recruited in response to expression of adhesion molecules and chemoattractants on activated cells (Lynch *et al.*, 2003). We believe that high levels of endotoxins measured in our study, in addition to other toxic aerosols such as ammonia, in the barn air may have activated expression of adhesion molecules and chemoattractants, such as IL-8, to promote recruitment of inflammatory cells (Vogelzang *et al.*, 2000; Donham *et al.*, 2002; Jagielo *et al.*, 1996b; Jagielo *et al.*, 1996a).

Lung sections from rats exposed to the barn air for 20 days contained more mucus-containing goblet cells in the airway epithelium compared to the controls, 1 day and five day exposure group. Chronic LPS exposure (Toward and Broadley, 2002) and many chronic respiratory diseases (Jackson, 2001) present mucus hyper-secretion as a hallmark feature of airway inflammation. Such an increased mucus production in the airways is associated with reduced airway caliber, occlusion of small airways, reduced FEV1 (Jackson, 2001), impaired gas exchange and compromised muco-ciliary clearance (Rogers, 2001). Our experiments do not identify the causative agent or the mechanisms of increase in mucus-containing goblet cells in the lungs of exposed rats. However, there are some possibilities. First, neutrophilic inflammation, such as one observed in the rats exposed to the barn air, has been shown to increase expression of epidermal growth factor and mucus synthesis (Kim *et al.*, 2004a). Second, elastase released from activated neutrophils is known to stimulate degranulation of goblet cells and secretion of mucus (Agusti *et al.*, 1998). Third, eosinophil recruitment, such as that observed in the lungs of 5- and 20-day exposed rats, is associated with goblet cell hyperplasia and increased

mucus production in asthma and chronic obstructive pulmonary disease (Williams, 2004; Siergiejko, 2003; Bocchino *et al.*, 2002). Lastly, chronic exposures to endotoxin, similar to those in our study, increase PAS-positive mucus cells (Vernooy *et al.*, 2002; Toward and Broadley, 2002). These data show a causal relationship among exposures to the barn air, increased AHR, neutrophil and eosinophil recruitment, activation of BALT and goblet cell hyperplasia in the exposed rats.

Although we observed higher levels of endotoxins in the barn air, our study does not address precise mechanisms of BALT activation in the exposed rats. We believe that the inflammatory and increased AHR outcomes in our study are due to a combined effect of exposure to endotoxins and other toxicants such as ammonia (Vogelzang *et al.*, 2000; Donham *et al.*, 2002; Sigurdarson *et al.*, 2004) in the barn air. Although swine barn air contains both gram-positive and gram-negative bacteria (Cormier *et al.*, 1990; Clark *et al.*, 1983), high levels of endotoxin in the study appear to be an indirect evidence for the presence of high-density of gram-negative bacteria in the barn air. We recorded higher levels of endotoxin in the barn air compared to those previously reported (Zejda *et al.*, 1994; Senthilselvan *et al.*, 1997b), which may be an outcome of husbandry practices as well as reduced ventilation in the winter season to conserve heat.

Our data show that, single and multiple exposures to endotoxin rich-swine barn air induce lung inflammation characterized by infiltration of inflammatory cells, increased mucus positive-epithelial cells and activation of BALT in 20-day exposed rats. Furthermore, single and five exposures increased AHR. Because the barn air, in addition to endotoxins, contains dust, ammonia, microorganisms, aeroallergens (Crook *et al.*, 1991), CO_2, molds (Duchaine *et al.*, 2000), H_2S (Chenard *et al.*, 2003), microorganisms and associated products such as bacterial cell wall, pig dander, fecal material and feed materials (Chang *et al.*, 2001), more *in vivo* animal studies and detailed characterization of the barn air are needed to precisely identify the causative agents and their respective contributions to lung dysfunction and specific interactions of host genome and the environment.

CHAPTER 5: ROLE OF TOLL LIKE RECEPTOR-4 IN LUNG INFLAMMATION FOLLOWING EXPOSURE TO SWINE BARN AIR*

5.1. Abstract

The authors tested a hypothesis that lung inflammation and airway hyperresponsiveness (AHR) induced following barn air exposure are dependent on Toll-like receptor 4 (TLR4) by exposing C3HeB/FeJ (intact *TLR4*, wild type [WT]) and C3H/HeJ (defective *TLR4*, mutant) mice either to the barn air (8 hours/day for 1, 5, or 20 days) or ambient air. Both strains of mice, compared to their respective controls, showed increased AHR following 5 exposures but dampened AHR after 20 exposures to show lack of effect of TLR4 on AHR. However, swine barn air induced lung inflammation, with recruitment of inflammatory cells and cytokine expression observed in WT but not in mutant mice. These data show different roles of TLR4 in lung inflammation and AHR in mice exposed to swine barn air.

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http://dx.doi.org/10.1080/01902140701807779
"The original publication is available at www.informaworld.com".

5.2. Introduction

The modern swine industry employs workers for eight-hour shifts in pig confinement buildings (Wenger, 1999; Wenger *et al.*, 2005). Unprotected exposure to barn air causes lung dysfunction characterized by decline in forced expiratory volume in one second (FEV1), increased IL-6 in serum and nasal lavage, IL-8 in nasal lavage and inflammatory cells in bronchoalveolar lavage fluid (BALF) (Larsson *et al.*, 1997; Larsson *et al.*, 1994; Dosman *et al.*, 2000). In addition to toxic gases such as hydrogen sulphide and dust, swine barn air contains significant concentrations of endotoxin (Charavaryamath *et al.*, 2005; Donham and Popendorf, 1985). It is believed that endotoxin in the barn air is central to lung dysfunction in the exposed barn workers (Vogelzang *et al.*, 1998). However, there are no data on the specific contributions of barn air endotoxin to airway hyperresponsiveness (AHR) and lung inflammation in the exposed subjects (Charavaryamath *et al.*, 2005; Larsson *et al.*, 1997).

Endotoxin induces activation of cell signaling through its interactions with Toll-like receptor 4 (TLR4) and MyD88 resulting in nuclear translocation of NF-κB and increased transcription of IL-1,-6,-8 and TNF-α (Takeda *et al.*, 2003; Aderem and Ulevitch, 2000). Polymorphism in TLR4 (Asp299Gly) is associated with a diminished response to acute inhalation of LPS or endotoxin and TLR4 antagonist E5564 suppresses physiologic and biologic responses to chronic LPS inhalation (Schwartz, 2001; Savov *et al.*, 2005). Mice with a non-functional TLR4 have been extensively used to study the role of TLR4 in endotoxin-induced immune responses (George *et al.*, 2001; Brass *et al.*, 2003). However, the role of TLR4 in barn air-induced AHR and lung inflammation remains unknown.

We tested a hypothesis that lung dysfunction following single or multiple exposures to endotoxin-rich barn air is mediated through TLR4. The data obtained by using C3HeB/FeJ (functional TLR4) and C3H/HeJ (defective TLR4) show that intact TLR4 is required for barn air induced lung inflammation but not for induction of AHR.

5.3. Materials and methods

5.3.1. Mice and treatment groups

The experimental protocols were approved by the University of Saskatchewan Campus Committee on Animal Care and experiments in accordance with guidelines of the Canadian Council on Animal Care. Specific pathogen-free, six-to eight-week-old male C3HeB/FeJ (WT, functional *TLR4* gene) and C3H/HeJ (mutant , natural mutation in *TLR4* gene, both acquired from Jackson laboratories, Bar Harbor, ME) mice were acclimatized for one week and had access to food and water *ad libitum*. Mice were randomly divided into eight groups (four groups each of WT and mutant; n=6 per group). All personnel involved in the analyses were blinded to the treatment groups.

5.3.2. Exposure to swine barn air

The exposure pattern, season, and the barn chosen for the current study were similar to our previous experiment (Charavaryamath *et al.*, 2005). Briefly, we exposed the mice by hanging the cages at an approximate height of 2 m from the floor in a commercial swine barn that had 60 dry sows and 3 boars. Both WT and mutant mice were exposed in parallel to barn air for 8 hours/day for 1 day, 5 consecutive days or for 4 cycles of 5 consecutive days (8 hours/day). Each cycle of 5 exposures was interrupted by keeping the mice in normal ambient air for 2 days. Control mice were kept in normal ambient air.

5.3.3. Barn air sampling for endotoxin analysis

The barn air was sampled twice weekly to determine endotoxin levels as described previously (Kirychuk *et al.*, 1998). Briefly, airborne barn dust was collected for 8 hours onto a preweighted, binder-free glass fibre inline filter (SKC, Edmonton, Canada) contained in the sampler hung at the level of mouse cages. Filters were desiccated before and after sampling and weighed before stored in 50-mL polypropylene centrifuge tubes at 4°C until endotoxin analysis (Kirychuk *et al.*, 1998). The exposed filters were washed individually in sterile centrifuge tubes with 10 mL of sterile pyrogen-free water, followed by incubation for 1 hour at room temperature in a sonicating water bath. Twofold

dilutions of the supernatant fluids were analyzed for gram-negative bacterial endotoxin using an end-point assay kit (Cambrex Bioscience, Walkersville, MD). The endotoxin standard (*Escherichia coli* O111:B4) was used in duplicate at 4 concentrations (0.1 to 1.0 endotoxin units [EU]/mL) in each assay to generate the standard curve. The lower detection limit was 0.1 EU/mL, which is equivalent to 1.0 EU per filter. The sampling time and flow rate were used to calculate the concentration of endotoxin in air (EU/m^3).

5.3.4. Viable microbial count

Air samples for viable microbial count was collected twice every week into a six-stage viable cascade impactor (Graseby, Smyrna, GA) with a vacuum pump at a rate of 0.67 ft^3/ min (18.86 L/min) for 20 seconds. Six medium plates of Tryptic Soy Agar with 5% sheep blood placed in the sampler were incubated at 37 ^oC for 18-24 hours, and the colonies were counted using the positive-hole method correcting for microbial coincidence. Bacteria associated with particles of 0.65-7μ (Stage 2-6 of the six-stage viable impact cascader) and >7 μ (Stage 1 of the cascader) were classified as respirable and non-respirable bacteria, respectively (ANDERSEN, 1958).

5.3.5. Measurement of airway hyperresponsiveness

AHR was measured within 2-3 hours after completion of barn or ambient air exposures in awake control and exposed-mice in response to increasing concentrations of methacholine (Mch) using head-out whole body plethysmography as described (Neuhaus-Steinmetz *et al.*, 2000; Charavaryamath *et al.*, 2005). Briefly, air was supplied to the head and body compartments of the plethysmograph and changes in respiratory airflow were monitored using a flow sensor linked via a preamplifier and A/D board (Kent Scientific, Litchfield, CT) to a computer-driven real-time data acquisition/analysis system (DasyTec USA, Amherst, NH). Each mouse was sequentially exposed to aerosols of saline alone and then increasing doses of Mch diluted in saline (0.75, 1.5 and 3.0 mg/mL) and Flow@50%Tve1 (lung airflow at 50% of the expiratory tidal volume) was noted for saline and each of the Mch concentrations.

5.3.6. Blood, bronchoalveolar lavage, tissue collection and processing

At the end of the exposure period, mice were euthanized (1 mg xylazine and 10 mg ketamine / 100g body weight, intraperitoneal) to collect blood with cardiac puncture and BALF with 3 ml of ice cold Hanks balanced salt solution. The total and differential leukocyte counts were performed using a haemocytometer and cytospins stained with Wrights stain, respectively. Lungs were perfused intratracheally at 25 cm H_2O pressure, removed following ligation of trachea and immersed in 4% paraformaldehyde for 18 hours. Three pieces from left lobe of the lung were embedded in paraffin for light microscopy. Five-micrometer thick sections were stained with Haematoxylin and Eosin stain for histopathological evaluation of pulmonary inflammation.

Histological signs of lung inflammation (septal infiltration of neutrophils, perivascular and peribronchiolar inflammation and perivascular edema) were evaluated by an observer blinded to the study design. Stained slides were coded and ten randomly selected fields (40X objective covering an area of 0.096 mm^2/field) were used to count septal neutrophils. When no neutrophils were seen, it was recorded as, "-", one to two cells as "+", three to six cells as, "+ +" and more than six cells as, "+ + +". Perivascular and peribronchiolar inflammation and perivascular edema was graded subjectively. Absence of inflammation and edema was recorded as, "-", minimal as, "+", moderate as, "+ +" and intense as, "+ + +".

5.3.7. Enzyme-linked immunosorbent assay (ELISA)

Concentration of TNF-α, IL-1β and IL-6 were measured using capture/detection antibody pairs and recombinant standards (R&D Systems, MN, USA), as described previously (Gordon *et al.*, 2000). Briefly, lung samples were homogenized in Hanks balanced salt solution (HBSS) (100 mg lung tissue/ ml of HBSS) containing protease inhibitor cocktail (100 µl/10ml; Sigma-Aldrich Co, MO, USA). ELISA plates were coated with capture antibody (over night at 4^oC), blocked with 1% bovine serum albumin (Sigma Aldrich, Canada) followed by addition of standards and samples (n=3,100 µl each in duplicates) and incubation over night at 4^oC. The plates were washed with PBS-Tween and incubated with detection antibody (60 minutes at 37^oC) followed by color detection reagents. The assays were read at 450 nm.

5.3.8. RNA isolation and quantitative real time reverse-transcriptase polymerase chain reaction (qRTPCR)

Total RNA was extracted from the lungs of mice by sequential extraction with TRIzol reagent (Invitrogen, Ontario, Canada) followed by treatment with RNase-free DNase (Qiagen, Ontario, Canada) and purification on RNeasy mini columns (Qiagen, Maryland, USA) following manufacturer's instructions. RNA integrity was confirmed by agarose gel electrophoresis and RNA was quantified by spectrophotometric analysis. The mRNA was reverse transcribed at 42^{o}C for 40 minutes by using StrataScript QPCR cDNA synthesis kit (Stratagene, USA) and universal oligo dT primer. This cDNA was used for qRTPCR analysis for the expression of tumor necrosis factor alpha (TNF-α; GenBank Accession No. NP_038721), interleukin 6 (IL-6, GenBank Accession No. NM_031168) and interleukin 1β (IL-1β; GenBank Accession No. NP_032387) genes using Brilliant SYBR Green QPCR kit (Stratagene, USA). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; GenBank Accession No. XR_004536) was used as the reference housekeeping gene. The reactions were performed using the primer pairs: 5'-ATGAGCACAGAAAGCATG-3' and 5'-GGGAACTTCTACTCCCTT-3' for TNF-α, 5'-ATGAAGTTCCTCTCTGCA-3' and 5'-TCTCATTTCCAGGATTTC-3' for IL-6, 5'-ATGGCAACTGTTCCTGAA-3' and 5'-GCCACAGCTTCTCCACAG-3' for IL-1β and 5'- TGCATCCTGCACCACCAACTG -3 and 5'-GGGCCATCCACAGTCTTCTGG -3 for GAPDH. A negative control reaction consisted of all the components of the reaction mixture except RNA. Real-time PCR analysis was performed using the MX 3005P LightCycler (Stratagene). The cDNA was denatured at 95°C for 5 minutes followed by amplification of the target DNA through 45 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 seconds and elongation at 72 °C for 30 seconds. Relative expression levels in various treatment groups versus control group were calculated after correction for expression of GAPDH using MxPro-MX3005P software.

5.3.9. Statistical analyses

All data were expressed as mean ± SD or median and inter-quatrile range. Group differences were examined for significance using two-way analysis of variance (ANOVA) with Tukey test as the *post hoc* test (Statistix, Analytical Software, Tallahassee, FL and SigmaStat for Windows Version 3.11, Systat Software Inc., San Jose, CA). We examined the effect of days of exposure to swine barn air (control, 1, 5 and 20 days) and mouse strains (wild-type or mutant) as two main factors along with the interaction effect between days of exposure and strain. Significance was established at $P<0.05$.

5.4. Results

5.4.1. Barn air characterization

The mean endotoxin concentration in the swine barn air for the period of exposure was 2357.80 ± 2525.16 EU/m^3 of air. The amount of endotoxin in control air samples from normal ambient air where control animals were housed was below the level of detection. The total viable aerobic bacterial counts (CFU/m^3 of air sampled) in the barn air during the exposure period are shown in Table 5.1. Air samples collected from the control environment did not yield any bacterial colonies.

5.4.2. Airway hyperresponsiveness

Inhalation of increasing concentrations of Mch decreased airflow (Flow@50%Tve1), indicating increased airway reactivity and broncho-constriction. Both WT and mutant mice responded similarly to increasing concentrations of Mch ($P=0.46$) and showed effect of exposure to barn air (days of exposure effect) on AHR compared to the controls ($P<0.05$). Five exposures induced higher AHR compared to the control, 1 and 20 exposures ($P<0.05$). Following 20 exposures, AHR in both WT and mutant mice was lower than the control, 1 day and 5 day groups (Figure 5.1A-B; $P<0.05$).

5.4.3. BALF total and differential cell counts

There were differences (days of exposure effect and interaction effect between days of exposure and strain) in total leukocyte numbers in BALF among the eight groups (Figure 5.2A; $P<0.001$). One day exposed WT mice showed higher BALF total leukocytes (Figure 5.2A), absolute neutrophils (Figure 5.2B), absolute macrophages (Figure 5.2C) and absolute lymphocytes (Figure 5.2D) compared to one day exposed mutant mice and all other WT groups ($P<0.001$). The total leukocyte, neutrophil and macrophage numbers in BALF did not differ among groups of control and exposed mutants ($P>0.05$). However, absolute lymphocyte numbers in BALF from 5 day exposed mutants were higher compared to 20 day exposed mutants (Figure 5.2D, $P<0.001$).

5.4.4. Blood cell counts

Baseline blood cell counts (control) between WT and mutants were not different (Figure 5.3A-C; $P>0.05$). Total blood leukocyte numbers were higher in one day exposed WT mice compared to WT control ($P<0.001$), WT 5 day exposed ($P=0.008$), WT 20 day exposed ($P=0.001$) and mutant 1 day exposed (Figure 5.3A; $P=0.02$). Blood absolute neutrophil numbers in 1 day exposed WT mice were higher compared to the control, 5 day and 20 day WT groups and mutant 1 day exposed mice. (Figure 5.3B; $P<0.001$). There was no difference in blood absolute neutrophils numbers among the mutants ($P>0.05$). None of the groups differed in their blood absolute lymphocyte numbers (Figure 5.3C; $P > 0.05$).

5.4.5. Histopathology

Semi-quantitative evaluation of histological signs of lung inflammation is summarized in Table 5.2. There was no inflammation in the lung sections of control (WT and mutant) mice (Figure 5.4A-B). The WT mice following one or five exposures but not 20 exposures to the barn air showed septal infiltration of neutrophils in lungs (Figure 5.4C). We have not observed any airspace neutrophils or lymphocytes in the lung section areas that we evaluated as these lungs were lavaged prior to processing them for histology. Interestingly, the lungs from mutant mice exposed to the barn air lacked or had

highly diminished leukocyte infiltration (Figure 5.4D). WT and mutant mice showed normal bronchiolar epithelium after single exposure (Figure 5.5A-B) but epithelium appeared to be damaged as it was detached from the basement membrane following five (Figure not shown) or 20 exposures (Figure 5.5C-D).

5.4.6. Cytokine expression in BALF

There were no differences among any of the groups in the concentrations of TNF-α, IL-1β and IL-6 in the BALF (Figure 5.6A-C; P>0.05).

5.4.7. Cytokine expression in lung homogenates

5.4.7.1. TNF-α

The TNF-α protein concentrations in the lung homogenates showed days of exposure effect (P=0.005) and interaction between days of exposure and strain effect (P=0.021). Mutant mice in the 5 exposure group showed higher TNF-α protein concentrations compared to the controls (P=0.003), 1 exposure (P=0.042) and 20 exposure (P=0.017) groups (Figure 5.6D). Within control animals, WT mice showed higher TNF-α protein concentrations compared to mutants (Figure 5.6D; P=0.003) but did not differ from any of the exposed WT groups.

TNF-α mRNA levels in the lung homogenates showed strain effect (P<0.001) and strain and days of exposure interaction effect (P=0.027). Following 5 (P<0.001) and 20 exposures (P=0.001), WT mice had higher expression of TNF-α mRNA compared to mutants (Figure 5.6G; P<0.05). While WT groups did not differ from each other in TNF-α mRNA levels, the mutant mice exposed for 5 days showed reduced mRNA levels compared to mutant controls (Figure 5.6G; P=0.017,).

5.4.7.2. IL-1β

The protein levels of IL-1β in both BALF and lung homogenates were not different while mRNA levels in the lung homogenates from 20 day exposed WT mice were significantly different from 20 day exposed mutants (P<0.001) as well as all other WT groups (Figure 5.6H; P<0.05).

5.4.7.3. IL-6

The protein levels in the lung homogenates for IL-6 did not differ among any of the groups (Figure 5.6.F). Single exposure to barn air caused an increase in IL-6 mRNA transcripts in the lung homogenates in WT mice compared to mutant and all other WT mice (Figure 5.6I; $P<0.05$).

Table 5.1. The total, respirable and non-respirable aerobic viable bacterial count (CFU/m^3 of air sampled) from the barn air.

Classification	**Viable aerobic bacterial count X 10^4 (CFU/m^3 of sampled air)***
Total	27.34 (11.07-45.78)
Respirable (0.65-7 μm)	24.11(6.82-37.73)
Non-respirable (≥ 7μm)	3.18 (1.00-16.60)

* Viable bacterial counts are expressed as median and inter-quartile range.

Table 5.2. Semi-quantitative evaluation of histological inflammation in lung sections.

Strain	Days of exposure	Septal neutrophils	Perivascular inflammation	Perivascular edema	Peribronchial inflammation
WT	Control	-	-	-	-
Mutant	Control	-	-	-	-
WT	1 day exposure	+ +	+ +	+ +	+ +
Mutant	1 day exposure	-	-	-	-
WT	5 day exposure	+ +	+ +	+ +	+ +
Mutant	5 day exposure	- to +	- to +	- to +	- to +
WT	20 day exposure	- to +	- to +	- to +	- to +
Mutant	20 day exposure	- to +	- to +	- to +	- to +

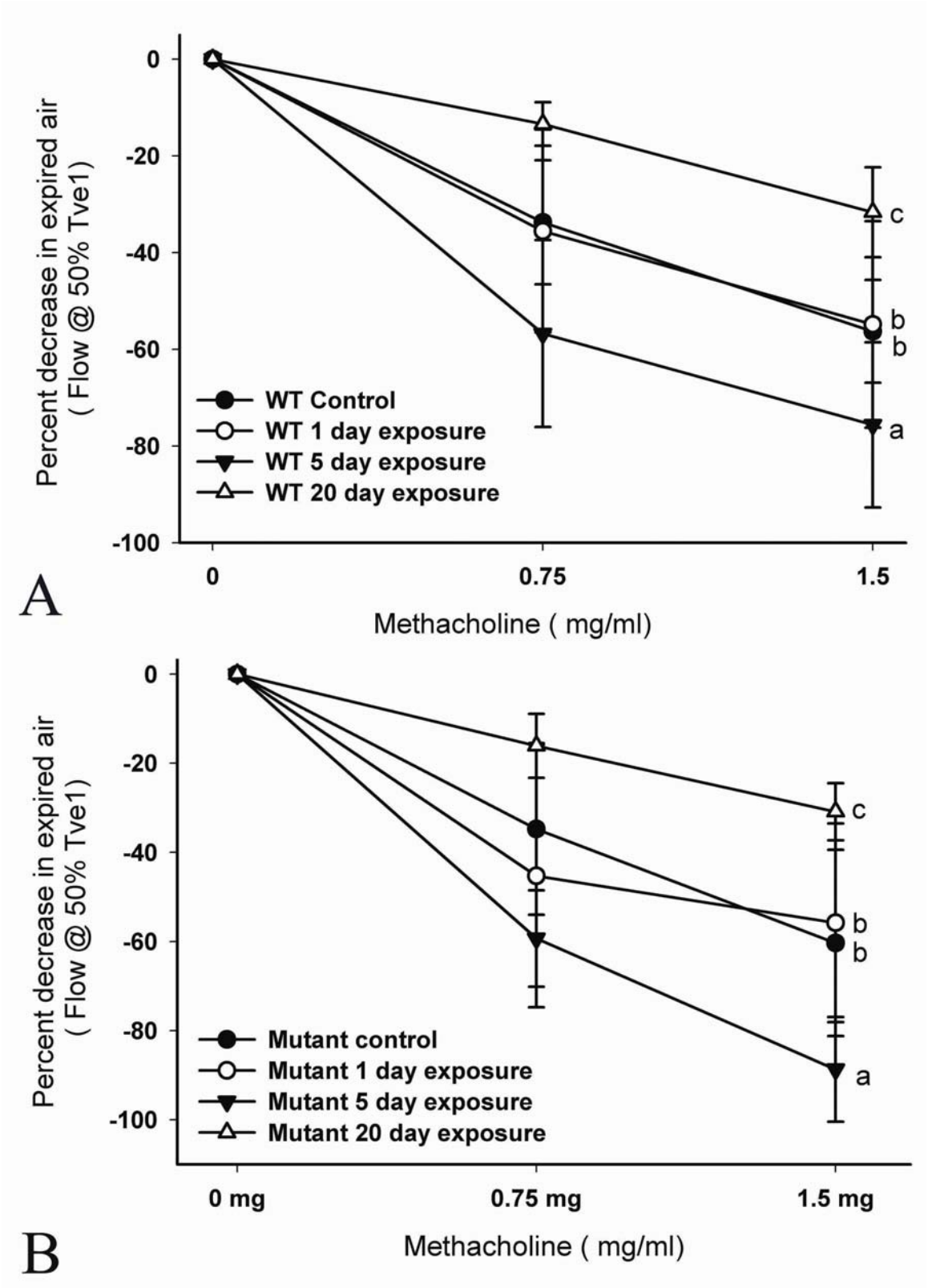
Percent decrease in expired air
(Flow @ 50% Tve1)
0
-20
-40
-60
-80
-100
WT Control
WT 1 day exposure
WT 5 day exposure
WT 20 day exposure
c
b
b
a
0
0.75
1.5
Methacholine (mg/ml)
A
Percent decrease in expired air
(Flow @ 50% Tve1)
0
-20
-40
-60
-80
-100
Mutant control
Mutant 1 day exposure
Mutant 5 day exposure
Mutant 20 day exposure
c
b
b
a
0 mg
0.75 mg
1.5 mg
Methacholine (mg/ml)
B

Figure 5.1. AHR.

AHR to methacholine challenge in mice was measured using a whole-body head-out plathysmograph. Both WT (A) and mutant (B) mice were similar in their AHR following barn or ambient air exposure ($P=0.46$). Increased and dampened AHR were seen following five and 20 day exposure, respectively ($P<0.05$). Groups bearing different superscripts (a, b and c) differ significantly from each other and those with same letter do not differ.

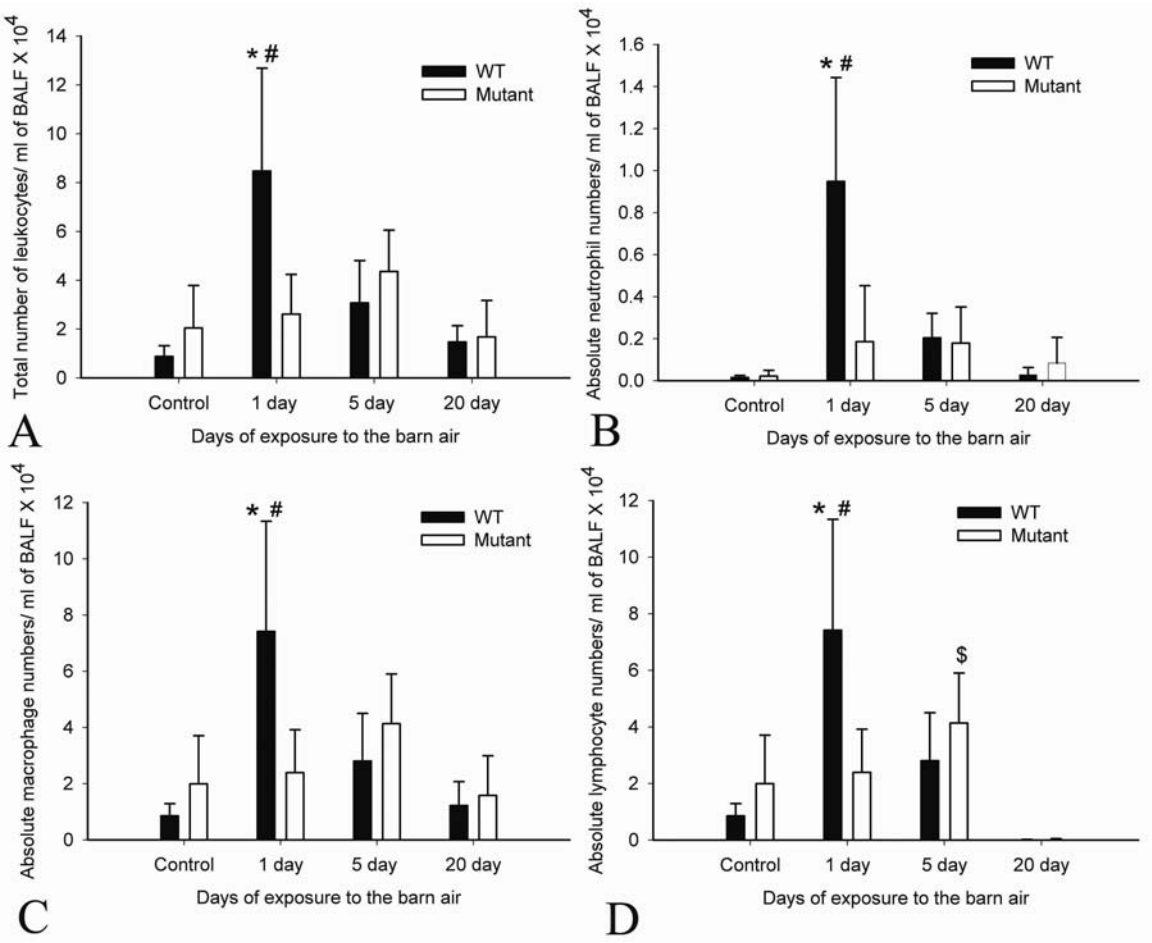

Figure 5.2. Total and differential leukocytes in the bronchoalveolar lavage fluid (BALF)

BALF total leukocytes were counted using haemocytometer and smears were differentiated with Wright's stain. One day exposed WT mice showed higher BALF total leukocyte numbers (A; $P<0.001$), absolute neutrophils (B), absolute macrophages (C) and absolute lymphocytes (D) compared to the mutants (*; $P<0.001$) and all other WT groups (#) whereas there were no differences among the mutant groups (A; $P>0.05$). Five day exposed mutants had higher numbers of BALF lymphocytes compared to the WT (D; $; $P<0.001$).

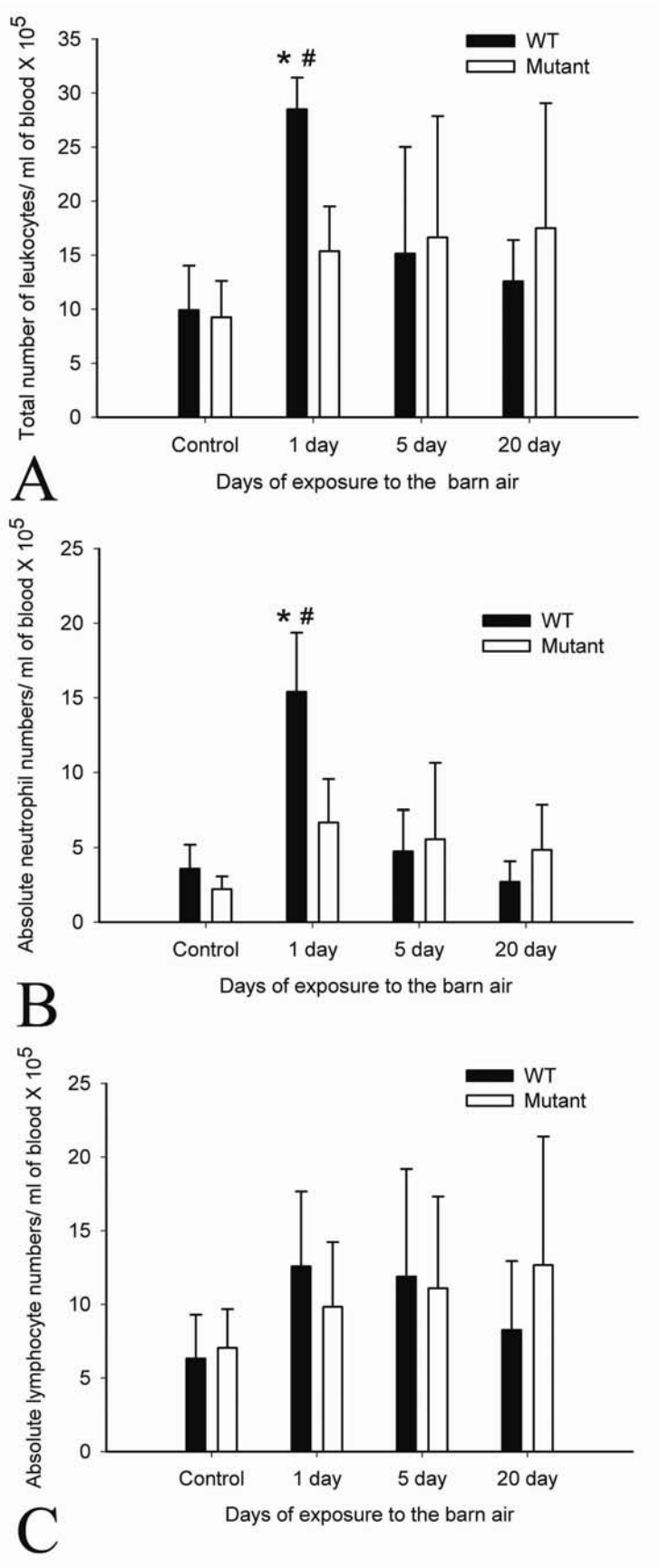
Total number of leukocytes/ ml of blood X 10^5
35
30
25
20
15
10
5
0
* #
WT
Mutant
Control
1 day
5 day
20 day
Days of exposure to the barn air
A
Absolute neutrophil numbers/ ml of blood X 10^5
25
20
15
10
5
0
* #
WT
Mutant
Control
1 day
5 day
20 day
Days of exposure to the barn air
B
Absolute lymphocyte numbers/ ml of blood X 10^5
25
20
15
10
5
0
WT
Mutant
Control
1 day
5 day
20 day
Days of exposure to the barn air
C

Figure 5.3.Total and differential leukocyte count in blood

Blood total leukocytes were counted using haemocytometer and smears were differentiated with Wright's stain. One day exposed WT mice showed higher blood total leukocytes (A) and absolute neutrophils (B) compared to one day exposed mutants (*; $P=0.002$) and other WT groups (#; $P<0.05$) whereas there were no differences among the mutant groups ($P>0.05$). Absolute lymphocyte numbers not different between WT and mutants (C; $P>0.05$).

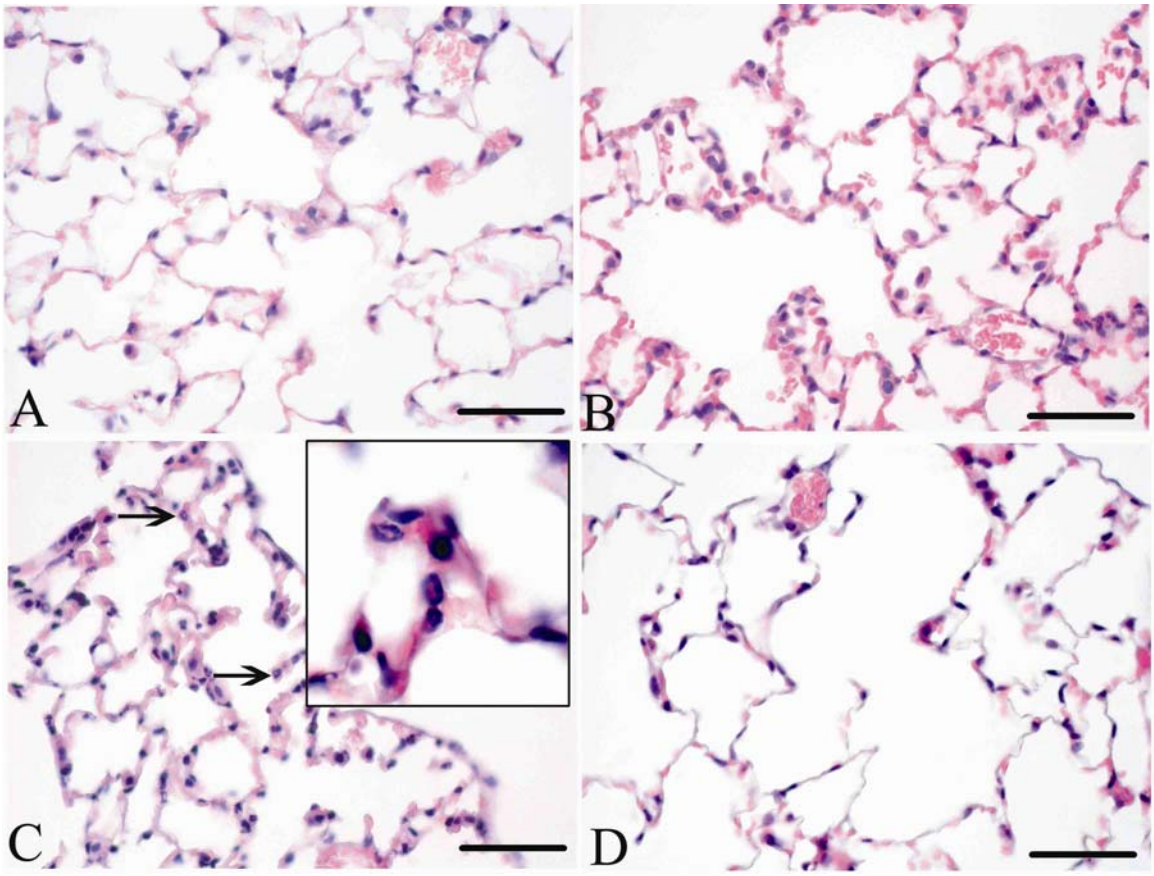

Figure 5.4. Histopathological evaluation of lung sections

Histopathological changes in the lungs of swine barn air exposed and control mice were evaluated using hematoxylin and eosin stained tissue sections. Lung sections from control WT (A) and mutant (B) strains showed no inflammatory cell infiltration and normal architecture of the organ. WT mice exposed for one day showed septal neutrophilic infiltration (C; arrows and inset) while lungs from mutant mice appeared to be normal (D). Original magnification: A to D: X400; Insets: X1000; scale bar=50 µm.

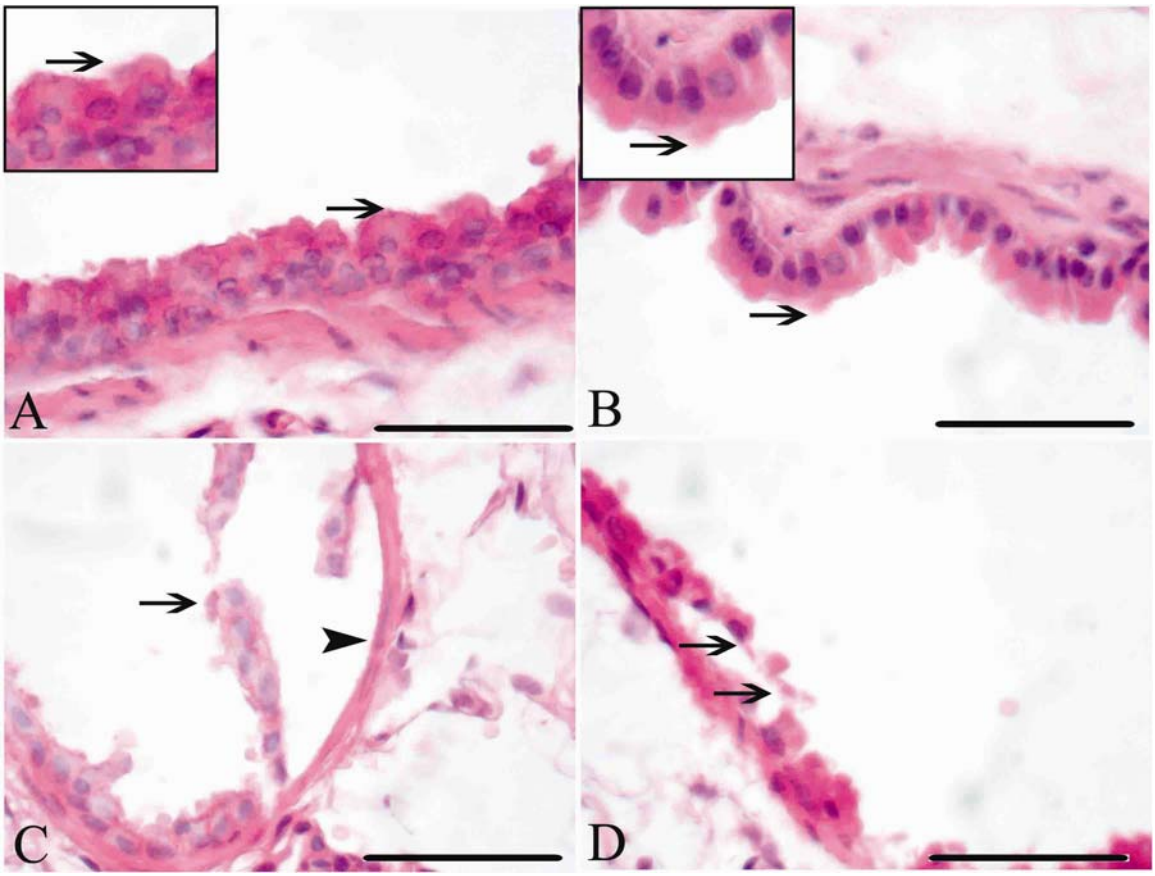

Figure 5.5. Histopathological evaluation of lung sections.

Histopathological changes in the lungs of swine barn air exposed and control mice were evaluated using hematoxylin and eosin stained tissue sections. Lung sections from both control WT (A) and control mutants (B) showed normal healthy airway epithelium whereas mice exposed to the swine barn air for 20 days showed damaged airway epithelium. The epithelium was detached (C, D, arrows) from the basement membrane (C, arrow head) in both WT (C) and mutant (D) mice. Original magnification: A-D: X400; scale bar =50 μm.

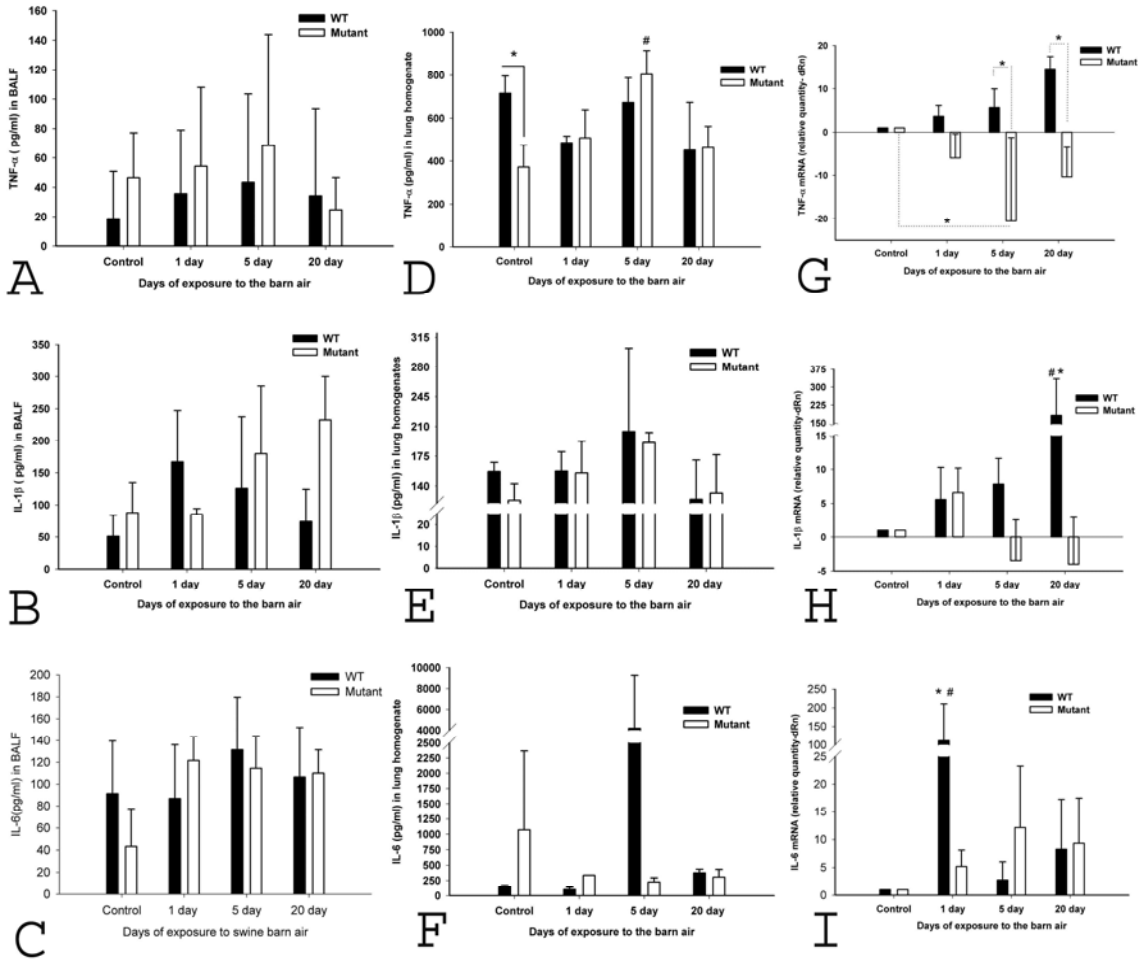

Figure 5.6. Quantification of cytokine protein (ELISA) and mRNA (real-time PCR) levels.

TNF-α protein levels in the BALF were similar among all the groups (A) whereas TNF-α protein concentrations in the lung homogenates from WT control mice were higher (D) compared to mutant controls (*P=0.003) and 5 day exposed mutant mice had higher TNF-α protein expression compared to other mutant groups (# P<0.05). TNF-α mRNA levels in the lung homogenates (G) were not different among WT mice although 5 day exposed mutants showed significantly reduced mRNA levels compared to mutant controls (*P=0.017). Following 5 (P<0.001) and 20 exposures (P=0.001), TNF-α mRNA levels were higher in WT compared to mutant mice. IL-1β protein concentrations in the BALF (B) and lung homogenates (E) were not different among any of the groups (P>0.05). IL-1β mRNA levels (H) in the lung homogenates of 20 day exposed WT mice were higher when compared to 20 day exposed mutants (*P<0.001) and all other WT animals (# P<0.05). IL-6 protein concentrations in the BALF (C) and lung homogenates (F) did not differ among all the groups (P>0.05). IL-6 mRNA levels in the lung homogenates (I) in one day exposed WT mice were increased compared to one day exposed mutants (*) as well as all other WT (#) groups (P<0.05).

5.5. Discussion

Barn air contains high concentrations of endotoxins in addition to many other toxic aerosols and is known to induce lung dysfunction (Charavaryamath *et al.*, 2005; Donham and Popendorf, 1985). TLR4 is central to endotoxin induced cell responses (Takeda *et al.*, 2003; Aderem and Ulevitch, 2000). Therefore, we used wild type and TLR4 mutant mice to clarify the role of endotoxin in barn air induced lung dysfunction. Our data reveal that lung inflammation but not AHR was suppressed in TLR4 mutant mice indicating a central role for endotoxin and TLR4 signaling in lung inflammation in mice exposed to barn air.

Five exposures to the barn air increased Mch-induced AHR which was dampened following 20 exposures in both WT and mutant mice compared to strain-matched controls. Although naïve human volunteers and rats show increased and diminished airway reactivity following single and multiple exposures to barn air, respectively (Malmberg and Larsson, 1993; Larsson *et al.*, 1994; Wang *et al.*, 1997; Palmberg *et al.*, 2002; Charavaryamath *et al.*, 2005), the mechanisms of such responses have remained poorly understood. We show for the first time that high concentrations of endotoxin in barn air and TLR4 signalling may not be the only players in induction of AHR in the exposed mice. These observations differ from the data that AHR observed following subchronic inhalation of *E. coli* LPS is dependent on a functional TLR4 and that TLR4 or LPS antagonists block lung responses to inhaled LPS (Lorenz *et al.*, 2001; Savov *et al.*, 2005). One of the reasons for the difference could be that barn air is a complex mixture of toxic molecules such as ammonia which may directly irritate airway epithelium to induce AHR in a TLR4-independent manner (Vogelzang *et al.*, 2000; Vogelzang *et al.*, 1997).Wearing a respirator capable of filtering both endotoxin and gas inside a swine confinement facilities could only reduce the inflammation but failed to influence the increase in bronchial responsiveness (Sundblad *et al.*, 2006). This shows complex regulation of AHR induced following exposure to swine barn air and that endotoxin may not be the only cause. In contrast to persistent and TLR4-dependent AHR observed in mice exposed daily for 5 days or 8 weeks to pure LPS (Cockcroft and Davis, 2006; Held and Uhlig, 2000; Toward and Broadley, 2000), we observed a dampening of AHR

following 20 exposures to barn air. The reasons for dampened AHR following 20 exposures are not clear except that it could be an adaptive response to one or more of the many toxic compounds in the barn air. Nevertheless, our data are the first to show that TLR4 does not influence AHR induced by single or multiple exposures to swine barn air.

Compared to controls, we report more lung inflammation including neutrophil migration into lungs of WT but not mutant mice following one or five exposures to the barn air. But following 20 day exposures, we see airway epithelial damage in both strains. Recent data has shown that TLR4 directly influences neutrophil migration into inflamed lungs (Andonegui *et al.*, 2003). Neutrophil recruitment is also regulated through a complex interplay of cytokines such as IL-6 and TNF-α, chemokines such as monocytes inflammatory protein (MIP-2) and adhesion proteins (Andonegui *et al.*, 2003; Burch *et al.*, 2006). Because expression of chemokines such as MIP-2 is regulated by IL-6 (Fenton *et al.*, 2002), we examined expression of IL-6, TNF-α and IL-1β in our study. In our experiments, WT mice compared to the mutants showed higher lung levels of IL-6 mRNA following a single exposure, higher lung levels of IL-1β mRNA following 20 exposures and higher levels of TNF-α mRNA following 5 or 20 day exposures to barn air. Other than higher levels of TNF-α protein in 5 day exposed mutant animals compared to all other mutant groups and WT control mice compared to mutant controls, there were no differences in protein expression of any of the cytokines in BALF or the lung homogenates. Although the mRNA and protein expression of cytokines do not correlate in our study, it is not unexpected because all of the mRNA may not be synthesized and secreted as the protein product due to translational or post-translational blocks. Furthermore, the processing of mRNA may occur at later time points, which we may have missed in our sampling design. Nevertheless, it is interesting to note that mRNA of at least one of the proinflammatory cytokines was higher at each of the exposure times (IL-6 after 1 exposure, TNF-α after 5 and 20 exposures and IL-1β after 20 exposures) in WT mice compared to the mutant mice. The higher expression of cytokines along with increased neutrophil recruitment in mice with functional TLR4 fits in with the data that TLR4 activation following exposure to LPS results in nuclear translocation of NFκB and expression of cytokines such as TNF-α and IL-6 that have established critical roles in lung inflammation (Bowie and O'Neill, 2000; Medzhitov *et al.*, 1997; Jeyaseelan

et al., 2004). Therefore, our data show that lung inflammation in mice exposed to pig barn air is dependent on a functional TLR4.

To our knowledge, this is the second study in which an animal model has been used to investigate barn air-induced lung dysfunction (Charavaryamath *et al.*, 2005). Because of high concentrations of endotoxin in the barn air, we compare the results with those obtained through application of pure LPS. It is significant to note that a barn is a cocktail of molecules such as ammonia, dust, bacterial DNA and endotoxins and we lack a complete understanding of their inflammatory properties either alone or in combination (Vogelzang *et al.*, 2000; Vogelzang *et al.*, 1997). Ammonia and dust in the barn air may directly irritate and damage the airway epithelium to induce inflammation and AHR (Burns *et al.*, 1985; Brautbar *et al.*, 2003) whereas endotoxin signaling through TLR4 may be a dominant pathway to induce lung inflammation. The role of other inflammatory molecules such as gram positive bacteria and bacterial DNA in the barn air needs to be explored further in relation to the biology of TLR2 and TLR9 (Takeda *et al.*, 2003; Aderem and Ulevitch, 2000).

Our data show that lung inflammation and AHR are differentially regulated in mice exposed to the barn air. Although lung inflammation induced following exposure to the barn air is dependent on TLR4 signalling, the AHR appears not to be. Interestingly, increased AHR observed following 1 or 5 exposures is dampened after 20 exposures. The mechanisms of AHR dampening need further investigation as well as the role of downstream signalling molecules such as MyD88. MyD88 acts as an adaptor molecule in TLR2, TLR4, TLR5, TLR7 and TLR9 pathways and is shown to be important in ovalbumin induced inflammation and airway reactivity (O'Neill and Bowie, 2007; Piggott *et al.*, 2005). Therefore, it would be interesting to study the role of MyD88 and other molecules such as Toll/IL-1 receptor-domain-containing adaptor inducing IFN-β (TRIF). Considering lack of lung inflammation in the mutant animals, our data also questions the contributions of barn air components such as ammonia and dust to lung inflammation and creates a need for further experiments.

CHAPTER 6: LUNG RESPONSES TO SECONDARY ENDOTOXIN CHALLENGE IN RATS EXPOSED TO PIG BARN AIR

6.1. Abstract

Swine barn air contains endotoxin and many other noxious agents. Single or multiple exposures to pig barn air induces lung inflammation and loss of lung function. However, we do not know the effect of exposure to pig barn air on inflammatory response in the lungs following a secondary infection. Therefore, we tested a hypothesis that single or multiple exposures to barn air will result in exaggerated lung inflammation in response to a secondary insult with *E. coli* LPS. We exposed Sprague-Dawley rats to ambient (N=12) or swine barn air (N=24) for one or five days and then half of these rats received intravenous *Escherichia coli* LPS (*E. coli* LPS) challenge, observed for six hours and then euthanized to collect lung tissues for histology, immunohistochemistry and ELISA to quantitatively analyze lung inflammation. Compared to controls, histological signs of lung inflammation were evident in barn exposed rat lungs. One day barn exposed rats challenged with *E. coli* LPS showed histological signs of lung inflammation and increased recruitment of granulocytes. Control, one and five day barn exposed rats with *E. coli* LPS challenge showed higher levels of IL-1β in the lungs compared to respective groups not given *E. coli* LPS. The levels of TNF-α in the lungs did not differ among any of the groups. Control rats without *E. coli* LPS treatment showed higher levels of TGF-β2 compared to controls treated with *E. coli* LPS. Based on these results, I conclude that exposure to pig barn air induces lung inflammation and a secondary *E. coli* LPS challenge following one day exposure exacerbates the lung inflammation. These data may have implications for the health of barn workers.

6.2. Introduction

Swine production is a major agricultural industry in Canada and employs many fulltime workers who may work in shifts of 8 hours/day and 5 days/week inside the confined barns (reviewed in (Charavaryamath and Singh, 2006)). Fulltime barn workers experience multiple-interrupted exposures to complex swine barn environments (Donham *et al.*, 1986; Cole *et al.*, 2000; Wenger, 1999; Wenger *et al.*, 2005). The swine barn environment is a heterogeneous mixture containing organic dust, various microbes, endotoxin and a number of gases such as ammonia, carbon dioxide, hydrogen sulphide and methane (Asmar *et al.*, 2001; Donham *et al.*, 1986; Donham and Popendorf, 1985). Therefore, despite appearing clean, the modern large scale barns can pose greater health risk to swine barn workers (Cormier *et al.*, 2000).

Previous work has shown that full-time swine farmers experience various respiratory symptoms, loss of lung function, increased AHR and airway inflammation (reviewed in (Charavaryamath and Singh, 2006)). Single (2-5 hour) experimental exposure of naïve human volunteers to barn environment has been shown to induce fever, malaise, drowsiness (Larsson *et al.*, 1994), bronchial responsiveness (Malmberg and Larsson, 1993) and lung inflammation with increased influx of neutrophils, lymphocytes, eosinophils and macrophages in broncholavelolar lavage fluid (BALF) as well as chemoattractants such as IL-8 (Larsson *et al.*, 1997; Larsson *et al.*, 1994; Cormier *et al.*, 2000). When compared to naïve volunteers, repeatedly exposed swine farmers demonstrate accentuated inflammatory and airway responses following a single experimental barn exposure (Larsson *et al.*, 1994; Palmberg *et al.*, 2002; Israel-Assayag and Cormier, 2002) to indicate a possible adaptation response.

Recently, we have used rat and mouse models to mimic occupational exposures of full-time barn workers and demonstrate that single or five exposures to the barn air can induce lung inflammation and AHR which were attenuated after 20 exposures to the barn (Charavaryamath *et al.*, 2005; Charavaryamath *et al.*, 2008). We have also reported that barn air induced lung inflammation but not AHR is dependent on TLR4 activation (Charavaryamath *et al.*, 2008). Previously, we have shown transient recruitment of pulmonary intravascular monocytes/macrophages (PIMMs) in rats at 48 hours after a

single 8-hour exposure to the barn air and that treatment of rats with *Escherichia coli* LPS (*E. coli* LPS) at 48 hours after the barn exposure results in robust lung inflammation (Gamage *et al.*, 2007). These data have linked recruitment of PIMMs in rats to enhancement of lung inflammation following a secondary challenge with *E. coli* LPS.

Exposure to barn induces lung inflammation and many systemic signs of illness (reviewed in (Charavaryamath and Singh, 2006)). Our previous data show that a secondary LPS challenge at 48 hours after a single 8 hour barn exposure induces more pronounced lung inflammation (Gamage *et al.*, 2007). However, we do not know the impact of a secondary LPS challenge at an earlier time point following a single barn exposure or following multiple exposures to the barn air. Because barn workers are exposed to a complex environment with different microbes, they may experience secondary bacterial infections which may alter lung inflammatory responses. Therefore, it is important to study the lung responses under such conditions. Hence, I used our previously characterized rat model (Charavaryamath *et al.*, 2005) to investigate the effects of secondary *E. coli* LPS challenge on rats exposed to barn air. I tested a hypothesis that a secondary *E. coli* LPS challenge will exacerbate the lung inflammation induced following single or multiple exposures to the barn air. My data showed that one day barn exposure and subsequent *E. coli* LPS challenge induces robust lung inflammation compared to one day barn exposed rats with increased granulocyte recruitment and IL-1β levels in the lung.

6.3. Materials and Methods

6.3.1. Rats and treatment groups

The animal experiment protocols were approved by Animal Research Ethics Board, University of Saskatchewan, Saskatoon, Canada and were conducted according to the Canadian Council on Animal Care Guidelines. Specific pathogen-free, six-week-old, male, Sprague-Dawley rats (Charles River Laboratories, Canada) were maintained in the animal care unit of Western College of Veterinary Medicine. Rats were randomly divided into six groups (n=6 each). All the personnel involved in collection and analyses of samples were blinded to the treatment groups.

6.3.2. Exposure to swine barn air and lipopolysachharide treatment

Our barn exposure procedure has been described (Charavaryamath *et al.*, 2005). Briefly, the rats were placed in the cages and the cages were hung from the barn ceiling approximately at a height of two meters from the floor. Rats were exposed either to ambient air (N=12) or to the barn air (N=24). Barn exposure was for a period of eight hours per day for one (N=12) or five days (N=12). Immediately following exposure to the barn or ambient air, one half of these rats (n=6/group) were euthanized to collect lung tissues while remaining half of the rats received a secondary challenge with *E. coli* LPS intravenously (1.5 µg/kg of body weight, Sigma-Aldrich, MD) 18 hours after completion of the barn exposure, observed for six-hours and euthanized to collect lung tissues for histology, immunohistochemistry and ELISA. Previously, we have demonstrated induction of lung inflammation with 1.5 µg/kg of body weight intravenous dose of *E. coli* LPS (Charavaryamath *et al.*, 2006; Gamage *et al.*, 2007).

6.3.3. Tissue collection and processing

Lung tissues were collected and processed as described before (Charavaryamath *et al.*, 2005; Charavaryamath *et al.*, 2006). Briefly, following euthanasia, three pieces from each lobe (left and right) of the lung were taken and fixed in 4% buffered-paraformaldehyde for 16-18 hours and embedded in paraffin. Haematoxylin and eosin stained five micron thick sections were used for histopathological evaluation of lung inflammation. Remaining lung tissue was snap frozen in liquid nitrogen and stored at – 80^{o}C until used.

Semi-quantitative evaluation of lung inflammation was performed as described before (Charavaryamath *et al.*, 2008). Briefly, histological signs of lung inflammation such as perivascular and peribronchiolar inflammation as well as perivascular edema were evaluated by an observer blinded to the study design. Stained slides were coded and randomly selected fields (40X objective covering an area of 0.096mm^2/field) were used for subjective grading of histological changes. Absence of inflammation and edema was recorded as, "-", minimal inflammation as, "+", moderate as, "+ +", intense as, "+ + +" and very intense as, "+ + + +".

6.3.4. Immunohistochemistry

Lung sections were processed for immunohistochemistry as described (Singh *et al.*, 2001). Briefly, the sections were deparaffinized, hydrated and incubated with 5% hydrogen peroxide for 30 minutes to quench endogenous peroxidase, treated with pepsin (2mg/ml in 0.01N HCl) for 45 minutes to unmask the antigens and blocked with 1% bovine serum albumin for 30 minutes. Sections were incubated with primary antibodies against TNF-α (1:50) , IL-1β (1:25), TGF-β2 (1:100) (all from Santa Cruz Biotechnology, Inc., CA), ED-1 (1:150, mouse anti rat CD68, AbD Serotec, NC) and anti-granulocytes (1:50, BD Biosciences, Mississauga, ON, Canada) followed by horseradish peroxidase (HRP)-conjugated respective secondary antibodies (1:150; DAKO A/S, Denmark). The reaction was visualized using a colour development kit (VECTOR - VIP, Vector laboratories, USA). Controls consisted of staining without primary antibody or with isotype matched immunoglobulin instead of primary antibody.

We used ED-1 and anti-granulocyte antibodies to detect and quantify septal macrophages and granulocytes in the lungs respectively. Previously, ED-1 antibody has been shown to recognize a lysosomal protein in rat monocytes/macrophages (Dijkstra *et al.*, 1985; Damoiseaux *et al.*, 1994), while anti-granulocyte antibody recognizes all types of granulocytes (van *et al.*, 1991) and has previously been used by our group (Gamage *et al.*, 2007). Following immunohistochemistry, stained slides (n=3/group) were coded and twenty randomly selected fields (40X objective covering an area of 0.09 mm^2/field) were used for counting ED-1 and anti-granulocyte positive cells in the lung septae.

6.3.5. Enzyme-Linked Immunosorbent Assay (ELISA)

We followed sandwich ELISA protocols to measure the concentrations of TNF-α, IL-1β and TGF-β2 using commercially available capture/detection antibody pairs and recombinant protein standards (TNF-α, BD Biosciences, ON, Canada and IL-1β and TGF-β2, R&D Systems, MN, USA) as described before(Gordon *et al.*, 2000; Charavaryamath *et al.*, 2006; Charavaryamath *et al.*, 2008). Briefly, lung samples were homogenized in Hanks balanced salt solution (HBSS) (100 mg lung tissue/ ml of HBSS) containing protease inhibitor cocktail (100 µl/ 10ml ; Sigma-Aldrich, St. Louis, MO,

USA). ELISA plates were coated with capture antibody (over night at 4^{o}C), blocked with 1% bovine serum albumin (Sigma Aldrich, Canada) followed by addition of standards and samples (n=3,100 µl each in duplicates) and incubation over night at 4^{o}C. The plates were washed with PBS-Tween and incubated with detection antibody (60 minutes at 37^{o}C) followed by color detection reagents and reading at 450 nm.

6.3.6. Statistical analyses

All data were expressed as mean ± SD. Group differences were examined for significance using two-way analysis of variance with Tukey Test as *post hoc* test (SigmaStat for Windows Version 3.11, San Jose, CA). Significance was established at $P<0.05$.

6.4. Results

6.4.1. Histopathology of lung sections

Semi quantitative evaluation of histological signs of lung inflammation is summarized in Table 6.1. Control rat lungs showed no signs of inflammation (Figure 6.1A) while rats treated with intravenous *E. coli* LPS alone and one or five day barn exposed rats with or without *E. coli* LPS testament showed lung inflammation characterized by peribrochiolar infiltration of neutrophils (Figure 6.1B), perivascular and peribrochiolar infiltration of inflammatory cells (Figure 6.1C-F) and perivascular edema (picture not shown).

6.4.2. Immunohistochemistical quantification of macrophages and granulocytes

There was no difference in the number of ED-1 positive cells in the lung septae among all the groups (Figure 6.2F; $P>0.05$). The number of granulocytes increased in the lung septae of one ($P=0.029$) or five ($P=0.051$) day exposed rats challenged with *E. coli* LPS when compared to one day exposed rats not treated with the LPS (Figure 6.3F).

6.4.3. Expression and quantification of IL-1β

Immunohistochemistry detected IL-1β in airway epithelium (Figure 6.4A-E), blood vessel wall, lung septa and occasionally in AM and quantification with ELISA revealed that ambient or barn air exposed rats (one or five exposures) that received *E. coli* LPS treatment showed significantly higher IL-1β levels compared to respective groups without *E. coli* LPS treatment (Figure 6.4F; $P<0.001$).

6.4.4. Expression and quantification of TNF-α

Immunohistochemistry detected TNF-α in airway epithelium (Figure 6.5A-E), blood vessel wall, lung septa and occasionally in AMs and quantification using ELISA revealed no difference among any of the groups (Figure 6.5F; $P>0.05$).

6.4.5. Expression and quantification of TGF-β2

Immunohistochemistry detected TGF-β2 in airway epithelium (Figure 6.6A-E), blood vessel wall, lung septa and occasionally in AMs and quantification using ELISA revealed that control rats without *E. coli* LPS challenge showed higher levels of TGF-β2 compared to control rats challenged with *E. coli* LPS (Figure 6.6F; $P=0.001$).

Table 6.1. Semi-quantitative evaluation of histological inflammation in lung sections.

Treatment groups	**Peri-vascular inflammation**	**Peri-bronchiolar inflammation**	**Peri-vascular edema**
Control	- to +	- to +	- to +
Control+LPS	+ + to + + +	+ + to + + +	+ to + +
1 day exposure	+ + to + + +	+ + to + + + +	+ + + +
1 day exposure+LPS	+ + to + + + +	+ + to + + + +	+ + to + + + +
5 day exposure	+ to + + +	+ to + + +	+ + to + + +
5 day exposure + LPS	+ + to + + +	+ +	+ + to + + +

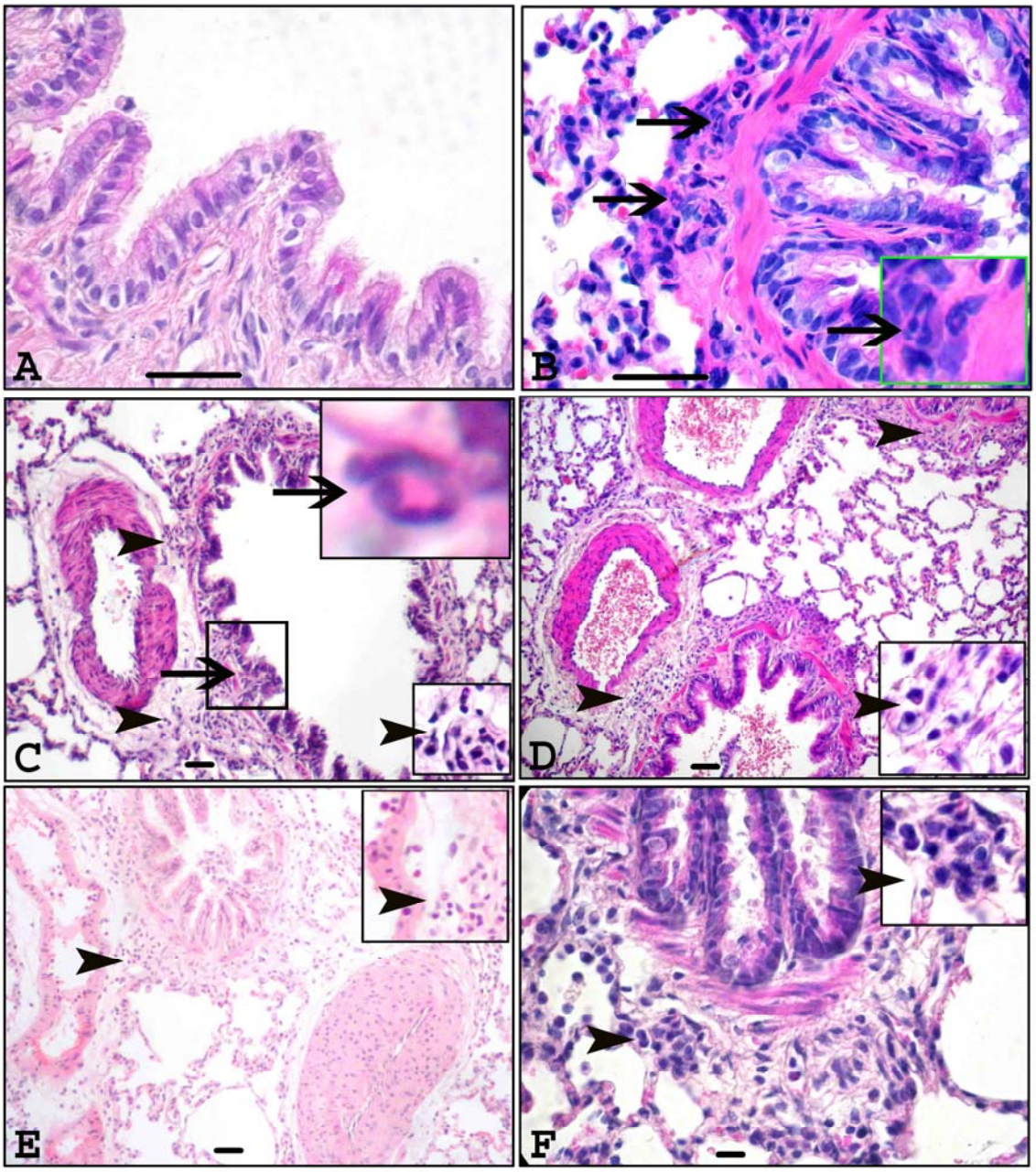

Figure 6.1. Histopathology of lung sections.

Histopathological changes in the lungs of rats exposed either to ambient (control) or swine barn air with or without *E. coli* LPS were evaluated using hematoxylin and eosin stained sections. Control rat lung tissues showed no inflammation and normal architecture of the organ (A) while rats treated with *E. coli* LPS (B), one day barn exposed rats without *E. coli* LPS (C) and with *E. coli* LPS (D), five day barn exposed rats with or without *E. coli* LPS (E and F respectively) showed peribronchiolar (arrows and inset, B) and septal neutophilic infiltration (arrows and inset, C), perivascular infiltration of leukocytes (arrowheads and insets, C-E), and peribronchiolar accumulation of

leukocytes (arrowhead and inset, F). *Original magnification* A- B: X400, C-F: X100 and micrometer bar = 50 μm.

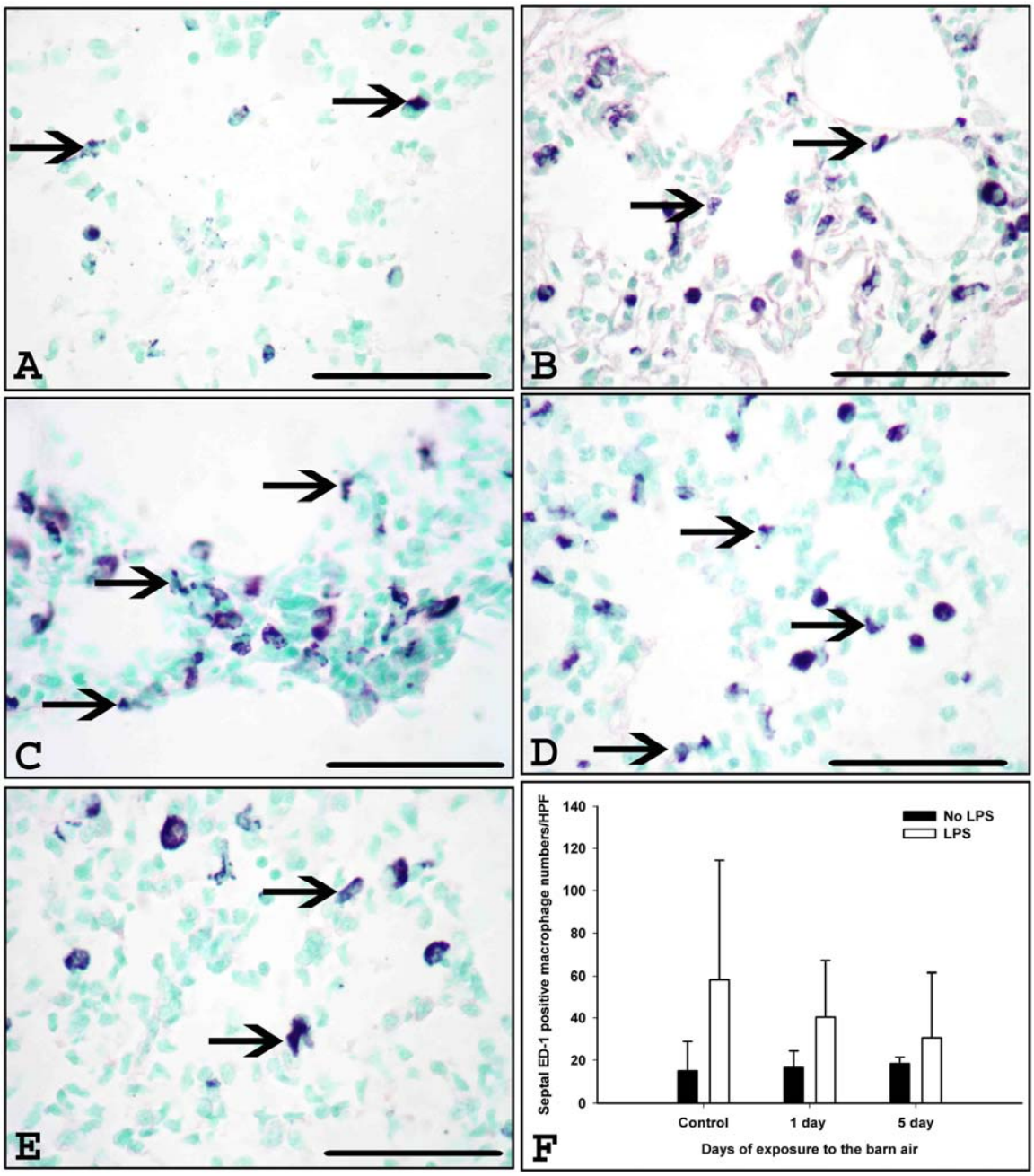

Figure 6.2. Immunohistochemical identification of monocytes/macrophages in the lung.

Monocytes/macrophages were stained using ED-1 antibody in the lung sections from control (A), *E. coli* LPS (B), one day (C-D) exposed rats without and with *E. coli* LPS challenge and five day (E) barn exposed rats (arrows). F: Quantification of septal monocytes/macrophages revealed no significant difference among any of the groups ($P > 0.05$). *Original magnification* A- F: X400 and micrometer bar = 50 µm.

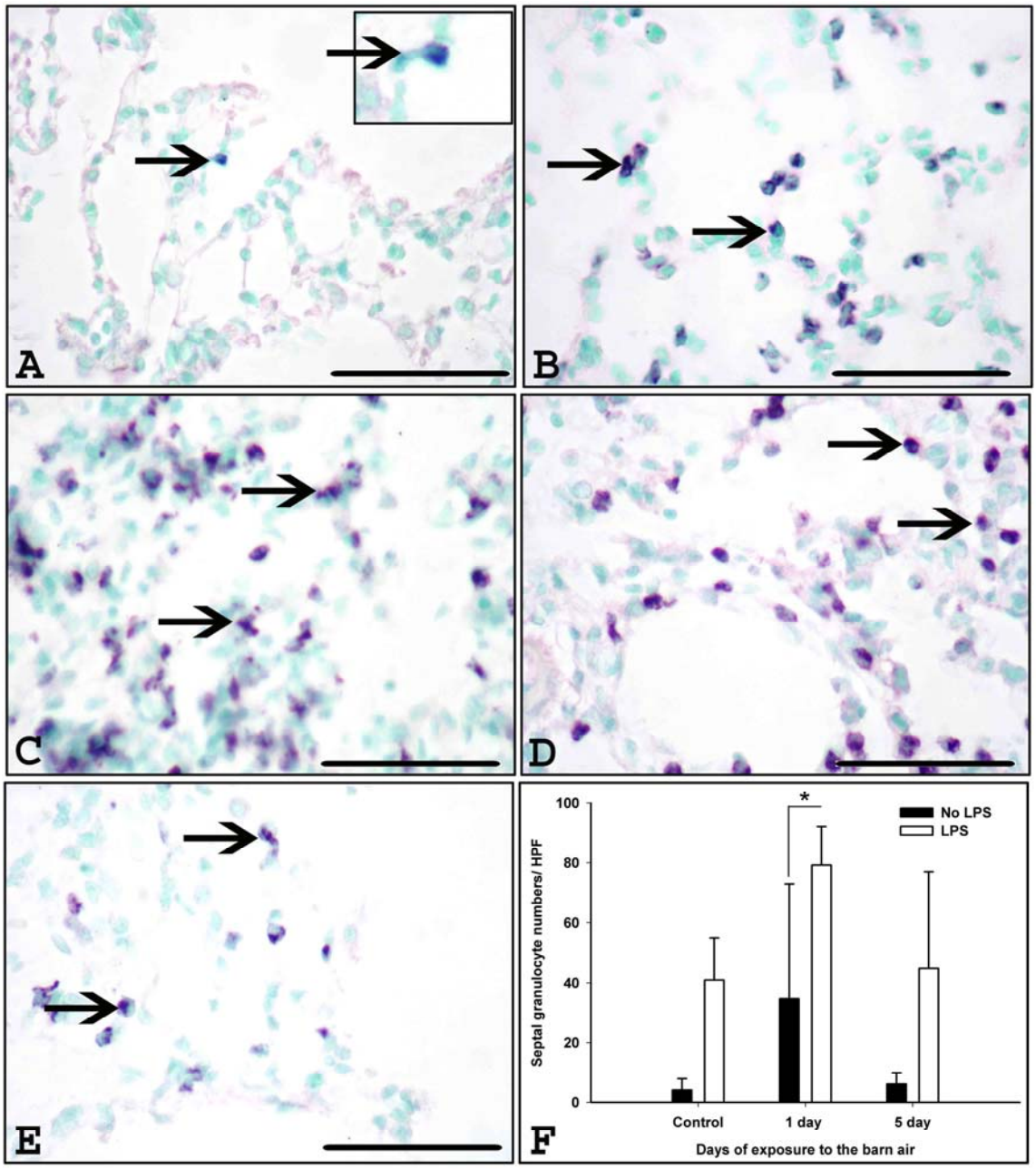

Figure 6.3. Immunohistochemical identification of granulocytes in the lung.

Granulocytes were stained using anti-granulocyte antibody in the lung sections from control (A, arrows and inset), *E. coli* LPS (B), one day (C-D) exposed rats without and with *E. coli* LPS challenge and five day (E) barn exposed rats (arrows, B-E). F: Quantification of septal granulocytes showed increased numbers in one day exposed rats with *E. coli* LPS challenge compared to one day exposed rats without *E. coli* LPS challenge (*, P=0.029) . Five day exposed rats with *E. coli* LPS challenge show a trend towards significant increase when compared to respective five day exposed rats without

E. coli LPS challenge (F, P=0.051) . *Original magnification* A- F: X400 and micrometer bar = 50 μm.

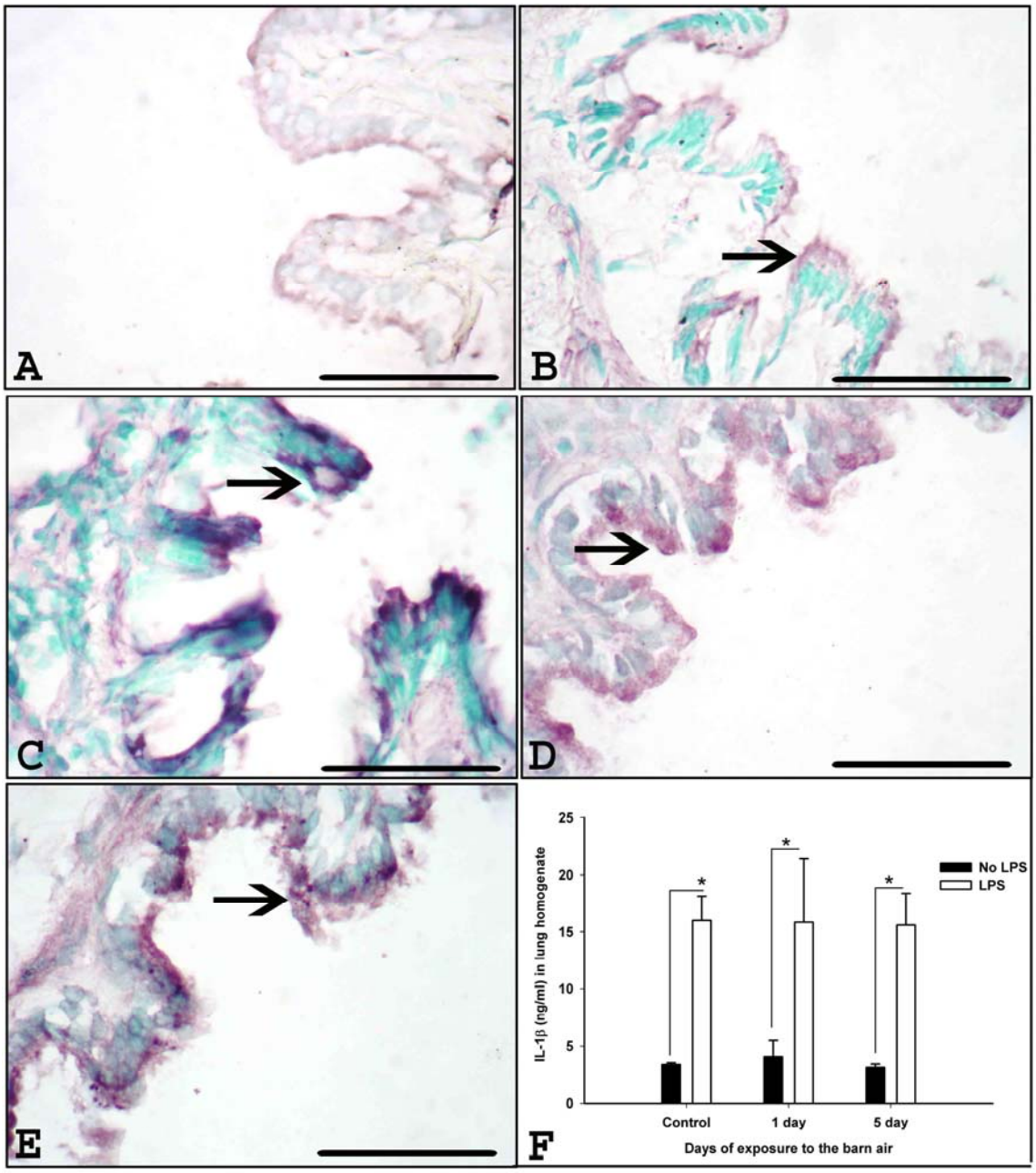

Figure 6.4. Expression and quantification of IL-1β in the lung.

Immunohistochemical expression of IL-1β was detected using anti-IL-1β antibody in the lung sections from controls (A-B), one day (C-D) exposed rats without and with *E. coli* LPS challenge respectively, five day exposed rats without *E. coli* LPS challenge (E) and with *E. coli* LPS challenge (picture not shown). IL-1β expession in the airway epithelium (arrows, A-E) is shown. F. Quantification of IL-1β protein using ELISA shows increased levels in rats that received *E. coli* LPS compared to respective groups of rats that did not receive *E. coli* LPS (*, $P<0.001$) . *Original magnification* A- F: X400 and micrometer bar = 50 μm.

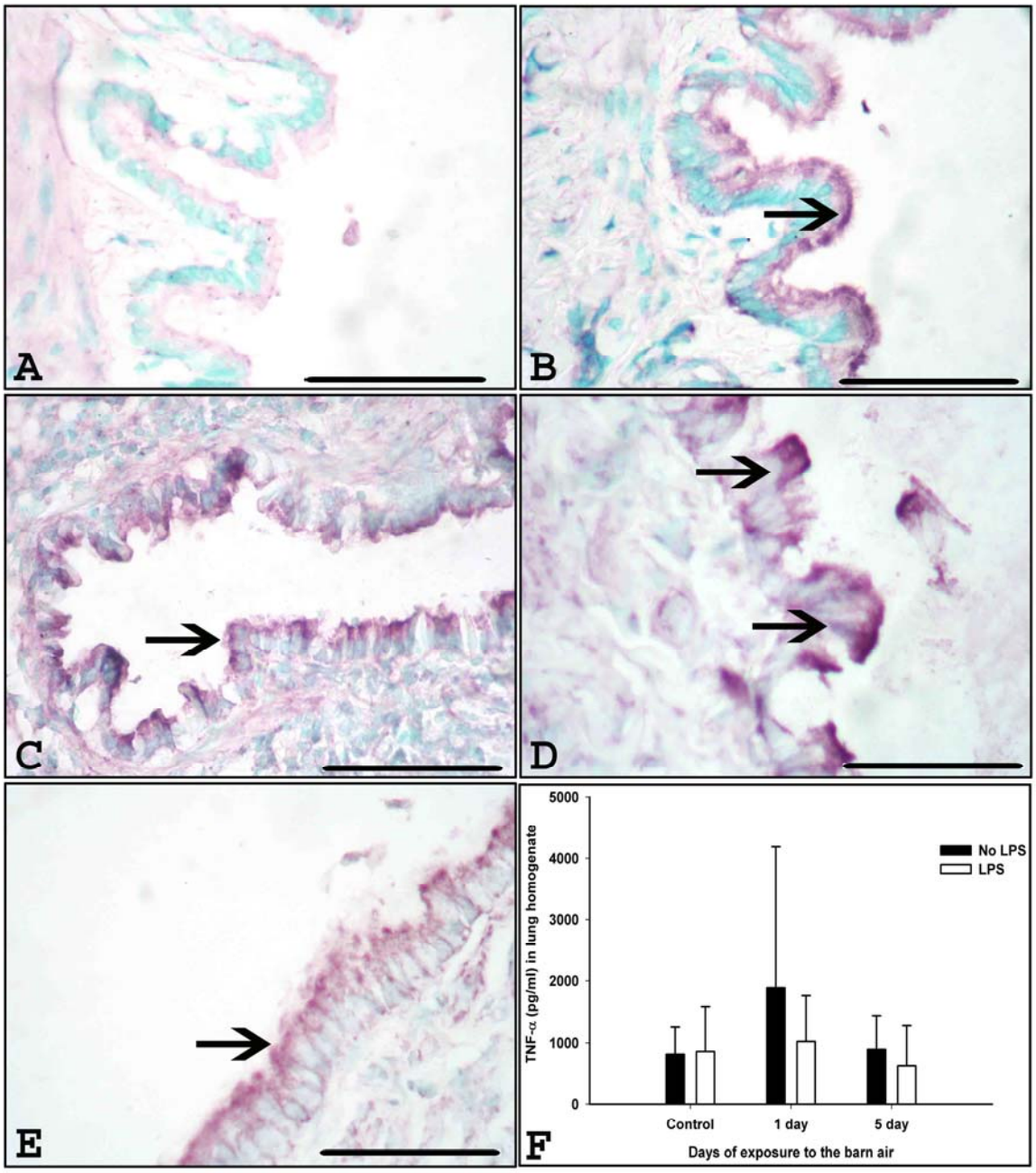

Figure 6.5. Expression and quantification of TNF-α in the lung.

Immunohistochemical expression of TNF-α was detected using anti-TNF-α antibody in the lung sections from controls (A-B), one day (C-D) exposed rats without and with *E. coli* LPS challenge respectively, five day exposed rats without *E. coli* LPS challenge (E) and with *E. coli* LPS challenge (picture not shown). TNF-α expression in the airway epithelium (arrows, A-E) is shown. F. Quantification of TNF-α protein using ELISA showed no significant difference among any of the groups (P>0.05). *Original magnification* A- F: X400 and micrometer bar = 50 μm.

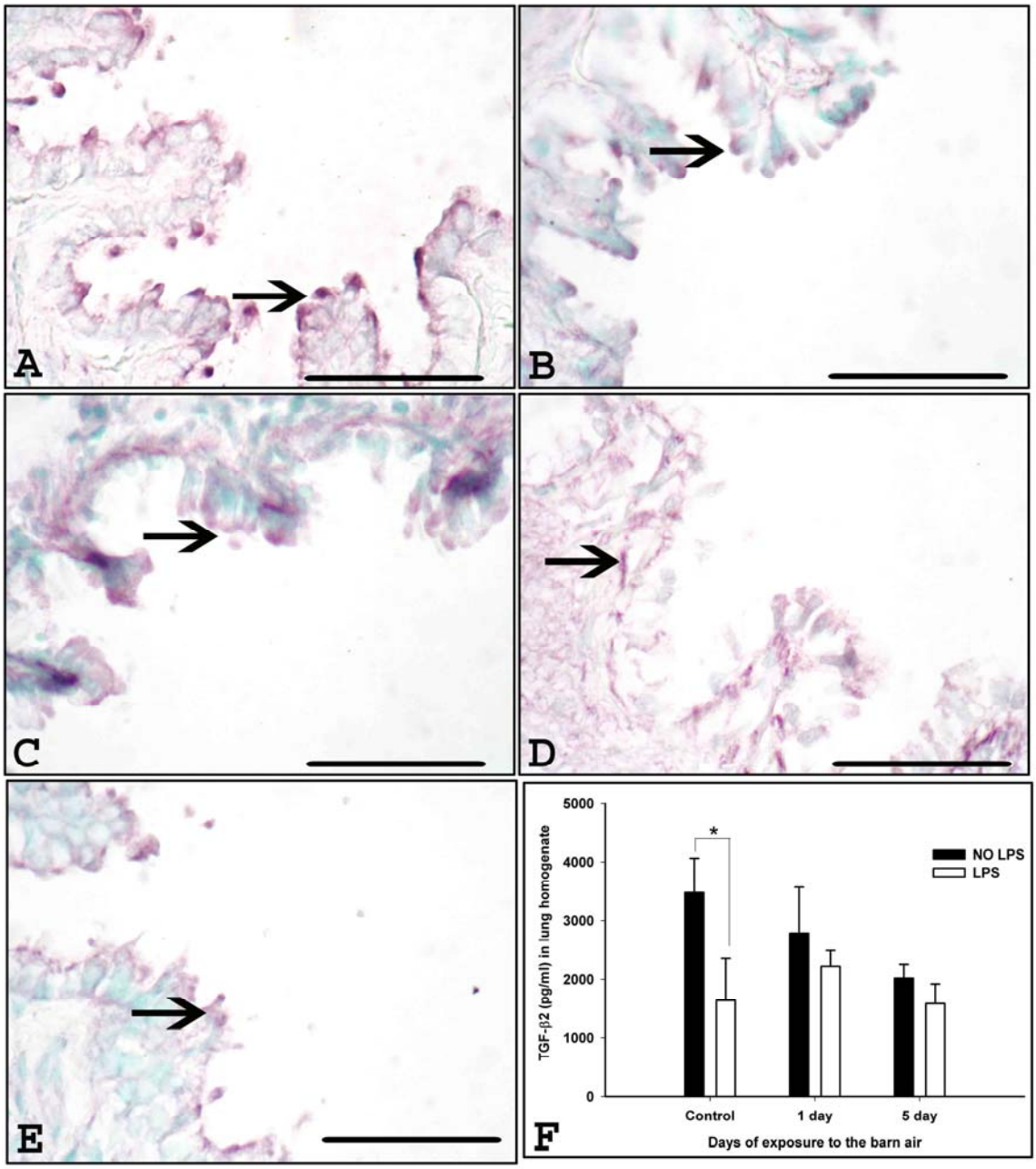

Figure 6.6. Expression and quantification of TGF-β2 in the lung.

Immunohistochemical expression of TGF-β2 was detected using anti- TGF-β2 antibody in the lung sections from controls (A-B), one day (C-D) exposed rats without and with *E. coli* LPS challenge respectively, five day exposed rats without *E. coli* LPS challenge (E) and with *E. coli* LPS challenge (picture not shown). TGF-β2 expression in the airway epithelium (arrows, A-E) is shown. F. Quantification of TGF-β2 protein using ELISA showed increased levels in control rats without *E. coli* LPS challenge compared to control rats with *E. coli* LPS challenge (*, P=0.001).*Original magnification* A- F: X400 and micrometer bar = 50 μm.

6.5. Discussion

In this study I report the effect of a secondary challenge with *E. coli* LPS on lung inflammation induced following exposure to swine barn air for one or five days with description of histological signs of lung inflammation, quantification of inflammatory cell recruitment and tissue expression and quantification of inflammatory cytokines. The data show that one or five exposures to swine barn air induce lung inflammation and following one day barn exposure and a secondary challenge with *E. coli* LPS, robust lung inflammation ensues with increased granulocyte recruitment in the lung septae. Rats from all the three groups following *E. coli* LPS challenge showed increased levels of IL-1β compared to rats from the respective groups without *E. coli* LPS challenge. Rats exposed to the ambient air (control rats) without *E. coli* LPS challenge showed higher levels of TGF-β2 when compared to *E. coli* LPS challenged controls rats.

In the current study I have demonstrated induction of lung inflammation following one or five days of barn exposure as before (Charavaryamath *et al.*, 2005; Charavaryamath *et al.*, 2008; Gamage *et al.*, 2007). The *E. coli* LPS challenge of rats at 18 hour post-exposure showed an increase in granulocyte recruitment in one day exposed rats while increased IL-1β levels was seen in both one day and five day exposed rats as well as control rats. The 18 hour post-exposure time was chosen for the secondary *E. coli* LPS challenge because significant recruitment of PIMMs does not occur at this time. Previous data showed significant PIMM recruitment at 48 hours after single exposure to the barn air and a secondary challenge with *E. coli* LPS during peak recruitment of PIMMs exacerbated lung inflammation (Gamage *et al.*, 2007). Although there were differences in lung inflammation following LPS challenge, there were no differences in granulocyte counts or IL-1β levels in barn exposed and *E. coli* LPS challenged rats compared to control rats treated with *E. coli* LPS. These observations raise the possibility of lack of priming effect due to barn exposure. It seems that induction of PIMMs is more critical to modification of lung inflammatory response (Gamage *et al.*, 2007) and not the direct effect of barn exposure itself. Nevertheless, we have demonstrated increased histological signs of lung inflammation following one day barn exposure and secondary

E. coli LPS challenge to indicate a certain risk for newly employed barn workers in the event of secondary microbial infections.

I observed that *E. coli* LPS treatment of both control and barn exposed rats resulted in higher expression of IL-1β in the lungs. But there were no differences in the lung expression of IL-1β in the control and the barn exposed rats challenged with LPS. Interestingly, the *E. coli* LPS treatment of control and barn exposed rats did not alter expression of TNF-α compared to the respective control rats without *E. coli* LPS challenge. IL-1β is a known early response cytokine in acute lung inflammation models and it is possible that we may have missed the first increase in IL-1β levels following barn exposures. Inflammatory cells such as monocytes/macrophages and neutrophils as well as endothelial cells and fibroblasts produce IL-1β which is a potent inducer of adhesion molecules that regulate neutrophil migration (Dinarello, 2000; Kasama *et al.*, 2005; Tosi, 2005; Williams, Jr. *et al.*, 1993; Parsey *et al.*, 1998). IL-1β also directly activates neutrophils through stimulation of mitogen activated protein kinases to result in increased super oxide anion production and respiratory burst in neutrophils (Yagisawa *et al.*, 1995; Suzuki *et al.*, 2001). IL-1β is also involved in inducing fever, increasing vascular permeability, production of IL-6 and leukocyte adherence to endothelium (Strieter *et al.*, 2002; Feghali and Wright, 1997). On the other hand, neutralization of IL-1β has proven protective and beneficial to the host (Standiford, 2000). Our data show that barn exposure does not dampen inflammatory response in the lungs and they remain capable of responding to secondary *E. coli* LPS challenge. This response is especially pronounced in one day exposed animals that showed more recruitment of granulocytes compared to the respective rats without *E. coli* LPS challenge. Neutrophils are the predominant granulocytes being recruited into the inflamed lung (Thorn, 2001) and are considered central to development of acute lung inflammation (Kinoshita *et al.*, 2000; Abraham, 2003). When neutrophils are primed by an initial injury, become activated, secrete increased amounts of oxygen radicals and cytokines (Williams, Jr. *et al.*, 1993).

Because lung inflammation is controlled by a complex network of both pro- and anti-inflammatory cytokines (Thacker, 2006), I examined the tissue expression and quantification of TGF-β2, a known anti-inflammatory cytokine with important roles in

tissue repair and remodeling (Hermiston, 2000). The data show reduced expression of TGF-β2 in LPS-treated control rats compared to control rats without *E. coli* LPS challenge. This observation indicates suppression of TGF-β2 levels in inflamed lungs possibly due to an active inflammatory reaction which is similar to previos reports of suppression of TGF-β2 exprssion in lungs of LPS-treated rats (Ayache *et al.*, 2002). We have reported similar data from rats exposed to barn and challenged with *E. coli* LPS at 48 hours later (Gamage *et al.*, 2007).

The data obtained in these experiments show no differences in lung inflammation between LPS-treated control and barn-exposed rats. These data are allude to the capability of lungs exposed to the barn air to respond to additional microbial challenges. As innate defense system comprised of TLR in the lungs is critical for initial defense response to microbes, my data indicate that one or five exposures to the barn air do not suppress innate immune system in the lungs. There is a need to evaluate lung responses to secondary challenge in animals exposed to the barn air for longer periods of time.

CHAPTER 7: EXPRESSION AND ACTIVITIES OF N-MYRISTOYLTRANSFERASE AND CALCINEURIN IN SWINE BARN AIR INDUCED LUNG INFLAMMATION

7.1. Abstract

Swine barn air is rich in endotoxin and also contains many other noxious agents. Therefore, full-time barn workers experience lung inflammation and decline in lung function. However, the mechanism of lung inflammation observed in swine farmers is not fully resolved. Since *N*-myristoyltransferase (NMT) and calcineurin (CaN) are involved in various cell-signaling pathways including inflammation, I sought to describe their expression and activities in lungs of rats exposed to swine barn air. I exposed Sprague-Dawley rats (n=6/group) to swine barn or ambient air for eight-hours and the third group of rats (n=6) was injected with *Escherichia coli* LPS intravenously and observed for six hours. All the rats were euthanized to collect lung tissues for histology, immunohistochemistry, enzyme assays and Western blotting for NMT and CaN. Compared to controls, both barn exposed and LPS treated rat lungs were inflamed. There was no difference in the activities of NMT and CaN among control, barn exposed and LPS treated rat lungs. Immunohistochemistry localized NMT and CaN in lung airway epithelium, blood vessel walls, alveolar macrophages and septa in all the three groups with increased intensity in LPS treated and barn exposed rats. Western blots revealed 55 and 60 kDa polypeptide bands corresponding to NMT and CaN, respectively, in the lung homogenates from all the groups. These results show an increased expression but not activities of NMT and CaN in acute lung inflammation.

7.2. Introduction

The lung is constantly exposed to a variety of inhaled antigens, irritants and blood borne microbial and non-microbial stimuli because of its chief role in respiration (Zhang *et al.*, 2000). Acute lung injury or inflammation (ALI) is induced by exposure to one or many of these exposures and is characterized by inflammatory cell influx, tissue edema, endothelial damage and increase in inflammatory cytokine expression. ALI can be induced with many stimuli, lipopolysachharide (LPS) or endotoxin is the most potent stimuli (Abbas and Lichtman, 2005b) and is ubiquitously found in many occupational work environments (Singh and Schwartz, 2005). Inhaled LPS or endotoxin mainly signals through TLR4 (Takeda *et al.*, 2003) to induce ALI characterized by rapid neutrophilic accumulation in the lung (Pauwels *et al.*, 1990), endothelial damage, increase in TNF-α and IL-1β and edema (Venaille *et al.*, 1989; Thorn, 2001). Clinically inhaled endotoxin or organic dust causes fever, headache, fatigue and malaise as well as chest tightness, cough, dyspnea, joint and muscle pain (for review(Thorn, 2001). Therefore, understanding the cell and molecular details of lung inflammation induced by pure endotoxin or endotoxin as an environmental contaminant is very important.

Modern, industrial scale swine production employs full-time barn workers (for review, see (Charavaryamath and Singh, 2006)) who are repeatedly exposed to the complex barn environment. The swine barn environment has many harmful agents such as ammonia, hydrogen sulphide, high levels of dust, microbes and endotoxins inside the confined buildings (Asmar *et al.*, 2001). Exposure to endotoxin in the barn air is a risk factor for the development of chronic respiratory diseases and annual decline in lung function (Senthilselvan *et al.*, 1997a; Zejda *et al.*, 1993). Swine barn air induces lung inflammation and airway hyperresponsiveness (Charavaryamath *et al.*, 2005). Our data also demonstrated regulation of lung inflammation but not airway hyperresponsiveness by TLR4 (Charavaryamath *et al.*, 2008). These data suggest a central role for endotoxin in pig barn air-induced lung inflammation although not in airway hyperresponsiveness. High concentrations of endotoxins in barn air appear to be the central cause of lung dysfunction and inflammation in barn workers (Charavaryamath *et al.*, 2005; Vogelzang *et al.*, 1998). Because of complex composition of pig barn air, there is a need to identify

the roel of signaling molecules other than TLR in lung inflammation following exposure to pig barn air.

N-myristoyltransferase (NMT) is a ubiquitous enzyme (for review (Selvakumar *et al.*, 2007) that catalyzes the co-translational, irreversible addition of a fatty acyl moiety to the amino terminus of proteins (*N*-myristoylation) and the process is important for many biological events. NMT belongs to the GCN5-related N-acetyltransferase super family of proteins and has been purified and characterized from various sources (Selvakumar *et al.*, 2007; Rajala *et al.*, 2000a; Boutin, 1997; Farazi *et al.*, 2001; Resh, 1999). This enzyme is believed to have a role in many diseases (Sharma, 2004; Selvakumar *et al.*, 2007). Increased NMT activity has been shown in early stages of rat and human colonic tumors and oral squamous cell carcinoma, ischemia-reperfusion injury and streptozotocin-induced diabetes (Magnuson *et al.*, 1995; Shrivastav *et al.*, 2007; Sharma, 2004). Myristoylation of TRIF-related adaptor molecule (TRAM) facilitates the transport protein to the plasma membrane where it is required for LPS responsiveness through the TLR4 pathway (Rowe *et al.*, 2006). Therefore, NMT appears to be an attractive therapeutic target in many disease conditions and LPS induced TLR4 signalling. Interestingly, there are no data on the expression and activities of NMT in lung inflammation induced by *Escherichia coli* LPS (*E. coli* LPS) or exposure to an occupational hazard such as swine barn air.

Calcineurin (CaN) is an eukaryotic Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase composed of a catalytic A subunit (59-62 kDa) and a regulatory B subunit (19 kDa) that plays a role in a number of cellular processes and Ca^{2+}-dependent signal transduction pathways (Rusnak and Mertz, 2000). Activated CaN directly binds to cytosolic transcription factor called nuclear factor of activated T cells resulting in its dephosphorylation and subsequent translocation into the nucleus to regulate cytokine, chemokine and cell-surface receptor gene expression (Bueno *et al.*, 2002; Macian *et al.*, 2001). CaN is shown to be involved in T-lymphocyte activation and cytokine signalling, neutrophil chemokinesis, lymphocyte degranulation, apoptosis and macrophage activation functions as well as in determining the outcome of neuroinflammatory processes (for review, see (Rusnak and Mertz, 2000; Hendey *et al.*, 1996; Fernandez *et al.*, 2007; Kim *et al.*, 2004b; Mitsuyama *et al.*, 2004; Boyd *et al.*,

2006)). CaN is known to negatively regulate TLR-mediated pathways through inhibition of the adaptor proteins MyD88 and TRIF, TLR proteins such as TLR2 and 4 but not 3 and 9 (Kang *et al.*, 2007). Despite these data, there is no information on the expression and activity of CaN in acute lung inflammation induced with *E. coli* LPS or a single eight-hour exposure to swine barn air.

Considering emerging roles of NMT and CaN in regulation of immune cells and expression of inflammatory mediators, I examined their expression and activities in the lungs from normal rats as well as those treated with *E. coli* LPS. Because of my interest in mechanisms of lung inflammation induced by exposure to swine barn air, I also examined expression and activities of NMT and CaN in a rat model of swine barn air induced lung inflammation. My data show increased expression of NMT and CaN in airway epithelium, blood vessel walls and septa of inflamed lungs. However, there were no differences in the activities of NMT and CaN among control, barn exposed and *E. coli* LPS treated groups.

7.3. Materials and methods

7.3.1. Materials

Polyclonal primary antibodies against NMT and CaN were produced and purified as described earlier (Rajala *et al.*, 2000b) and horseradish peroxidase (HRP)-conjugated rabbit secondary antibody was obtained from Dako Demnark A/S (Glostrup, Denmark).The color development kit was procured from Vector Laboratories Inc., (Burlington, Ontario, L7N 3J5, Canada) while nitrocellulose membrane was purchased from Bio-rad Laboratories (Mississauga, Ontario, Canada). The chemiluminescence reagents were obtained from Perkin Elmer Life Sciences (Boston, MA, USA) while *E. coli* LPS was obtained from Sigma-Aldrich (St. Louis, MD, USA).

7.3.2. Rats and treatment groups

The experimental protocols were approved by the Animal Research Ethics Board, University of Saskatchewan, Saskatoon, Canada and were conducted according to the Canadian Council on Animal Care Guidelines. Specific pathogen-free, six-week-old, male, Sprague-Dawley rats (Charles River Laboratories, Canada) were maintained in the

animal care unit of Western College of Veterinary Medicine. Rats were randomly divided into three groups (n=6 each). All personnel involved in collection and analyses of samples were blinded to the treatment groups.

7.3.3. Exposure to swine barn air and lipopolysachharide treatment

The barn exposure procedure has been described previously (Charavaryamath *et al.*, 2005). Briefly, the rats (n=6/group) were kept in the barn for 8 hours in cages that were hung from the ceiling approximately 2 meters from the floor. Another six rats were exposed to the ambient air, treated with *E. coli* LPS intravenously (1.5μg/kg bodyweight) and euthanized at 6 hours post-treatment. Control rats were only exposed to the ambient air and euthanized similarly.

7.3.4. Tissue collection and processing

These procedures have been described previously (Charavaryamath *et al.*, 2005; Charavaryamath *et al.*, 2006). Following euthanasia, three pieces of tissue were taken from each lung and fixed in 4% buffered-paraformaldehyde for 16-18 hours for light microscopy. Haematoxylin and eosin stained sections were used for histopathological evaluation of lung inflammation. Remaining lung tissue was snap frozen in liquid nitrogen and stored at – 80 ^{o}C until used.

7.3.5. Immunohistochemistry

Lung sections were processed for immunohistochemistry as described (Singh *et al.*, 2001). Briefly, the sections were deparaffinized, hydrated and incubated with 5% hydrogen peroxide for 30 minutes to quench endogenous peroxidase, treated with pepsin (2mg/ml in 0.01N HCl) for 45 minutes to unmask the antigens and blocked with 1% bovine serum albumin for 30 minutes. Sections were incubated with polyclonal primary antibodies against NMT and CaN (1:150; raised in rabbits) (Selvakumar *et al.*, 2005; Lakshmikuttyamma *et al.*, 2006) followed by HRP-conjugated rabbit secondary antibodies (1:150). The reaction was visualized using VECTOR-VIP colour development kit. Controls consisted of staining without primary antibody or with isotype matched immunoglobulin instead of primary antibody.

7.3.6. Semi-quantification of immunohistochemical expression of CaN and NMT

Rat lung sections immunohistochemically stained for CaN and NMT were evaluated by an observer blinded to the study design. Stained slides were coded and randomly selected fields (40X objective covering an area of 0.096 mm^2/field) were used to subjectively grade the staining intensity in the airway epithelium, blood vessels and lung septae. Absence of staining was recorded as, "-", minimal staining as, "+", staining moderate as, "+ +" and intense staining as, "+ + +".

7.3.7. Preparation of tissue extracts

All procedures were carried out at 4^oC, unless otherwise stated. Lung tissues were homogenized in 100 mM Tris-HCl, pH 7.4, containing 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mg/mL leupeptin. The crude homogenate was centrifuged for 30 min at 10,000 g and the supernatant was filtered through glass wool and used for subsequent analysis.

7.3.8. Determination of NMT activity

[^{3}H]Myristic acid (39.3 Ci/mmol) was obtained from NEN Life Science Products. *Pseudomonas* acyl CoA synthetase, coenzyme A, were obtained from Sigma-Aldrich Canada. The peptide based on the NH_2-terminal sequence of the type II catalytic subunit of cAMP-dependent protein kinase (GNAAAAKKRR) was obtained from Alberta Peptide Institute, University of Alberta, Edmonton, Canada. The NMT activity was measured as previously described (King and Sharma, 1991). For the standard enzyme assays, the reaction mixture contained 0.4 μM [^{3}H]myristoyl-CoA, 50 mM Tris-HCl, pH 7.8, 0.5 mM EGTA, 0.1% Triton X-100, 500 μM of cAMP-dependent protein kinase (GNAAAAKKRR) as a synthetic peptide and cell lysate as NMT source in a total volume of 25 μl. The reaction was initiated by the addition of radiolabeled [^{3}H]myristoyl-CoA and incubated at 30 °C for 30 min. The reaction was terminated by spotting aliquots of incubation mixture onto P81 phosphocellulose paper discs and drying them under a stream of warm air. The P81 phosphocellulose paper discs were washed in three changes of 40 mM Tris-HCl, pH 7.3, for 90 min. The radioactivity was quantified in 7.5 ml of Beckman Ready Safe Liquid Scintillation mixture using a Beckman Liquid Scintillation

Counter. One unit of NMT activity was expressed as 1 pmol of myristoyl-peptide formed per min per mg protein.

7.3.9. CaN assay

CaN activity was assayed using p-nitrophenylphosphate (pNPP) as a substrate as described previously (Pallen and Wang, 1983; Lakshmikuttyamma *et al.*, 2004). The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 1 mM Ni^{2+}, 5 μg CaM, 3.4 mM pNPP and CaN in a total volume of 1.0 mL. The mixture was incubated at 30^{o}C for 30 min. The reaction was initiated by the addition of pNPP and terminated by the addition of 75 mM K_2HPO_4. The pNPP hydrolysis was quantified by the increase in absorbance at 405 nm. One unit of phosphatase activity was defined as the amount of dephosphorylation resulting in an optical density of 0.1 at 30^{o}C after incubation for 30 minutes.

7.3.10. Western blotting analysis of NMT and CaN

Western blotting was performed as per the procedure described (Towbin *et al.*, 1979). Briefly, lung tissues were ground in liquid nitrogen and lysed in protein lysis buffer. After 20 minutes of incubation on ice, the homogenates were centrifuged at 20,000 g for 15 minutes; supernatants containing the protein extracts were transferred to clean tubes and stored at -80°C until analysis. The protein content in the extracts was quantified using the Bradford dye-binding assay (Bio-Rad laboratories, Hercules, USA) with BSA as a standard (Bradford, 1976). An equal amount (50 μg) of protein from lung homogenates was resolved on 10% (for NMT) or 12% (CaN) SDS-PAGE gel and electro-blotted onto nitrocellulose membranes. The membranes were blocked at room temperature with 5% (w/v) skimmed milk in TBS buffer, pH 7.4 containing 0.2% (v/v) Tween-20 (TBST) followed by incubation either with the anti-NMT or anti-CaN polyclonal primary antibodies at 4 °C overnight. Membranes were washed in TBST and incubated for 60 minutes with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000). Antigen-antibody complexes were visualized using chemiluminescence reagents and exposed to Kodak X-OMAT Blue XB-1 film for detection of immunoreactive bands.

7.3.11. Statistical analyses

All data were expressed as mean ± SD and group differences were examined for significance using one-way analysis of variance (SigmaStat Version 3.11, Systat Software Inc., Chicago, IL 60611). Significance was established at $P < 0.05$.

7.4. Results

7.4.1. Histopathology of lung sections

Control rat lung tissues showed no inflammation (Figure 7.1A and B) while rats treated with *E. coli* LPS (Figure 7.1C and D) and one day barn exposed rats (Figure 7.1E and F) showed septal neutophilic infiltration (C and E), leukocyte adherence to the endothelium of a blood vessel (D), perivascular infiltration of leukocytes (D) and peribronchiolar accumulation of neutrophils (F).

7.4.2. Semi-quantitative analysis of immunohistochemical expression of NMT and CaN

NMT and CaN were localized in the lung sections from rats from all the groups. Both NMT and CaN were expressed in airway epithelium (Figure 7.2), blood vessel wall (Figure 7.3), lung septae and alveolar macrophages (Figure 7.4). Semi-quantitative evaluation of immunohistochemical expression showed increased staining in airway epithelium, blood vessels and lung septae but not alveolar macrophages from rats exposed to *E. coli* LPS or to the pig barn air for one day (Table 7.1).

7.4.3. Expression and activities of NMT and CaN

Quantitative enzyme assays revealed no statistical difference in the activities among the three groups for both NMT and CaN (Figure 7.5; $P>0.05$). Through Western blotting, I detected both NMT and CaN in the lung homogenates from all the three groups. Polyclonal antibodies against NMT and CaN detected bands approximately of 55 kDa and 60 kDa corresponding to NMT and CaN, respectively (Figure 7.6A and B). Compared to control, NMT protein expression was 15-fold higher in *E. coli* LPS treated group and 50-fold higher in 1 day exposed group. When compared to 1 day exposed

group, CaN expression is 10 fold higher in control and 25 fold higher in *E. coli* LPS treated group.

Table 7.1. Semi-quantitative evaluation of NMT and CaN expression in lung sections.

	NMT			**CaN**		
Groups	**Airway epithelium**	**Blood vessels**	**Lung septa**	**Airway epithelium**	**Blood vessels**	**Lung septa**
Control	+ to ++	+	+ to ++	+	+	+
Control+ *E. coli* LPS	++ to +++	++ to +++	++ to +++	++ to +++	++	++ to +++
1 day exposure	++ to +++	+ to ++	++	++	++	++ to +++

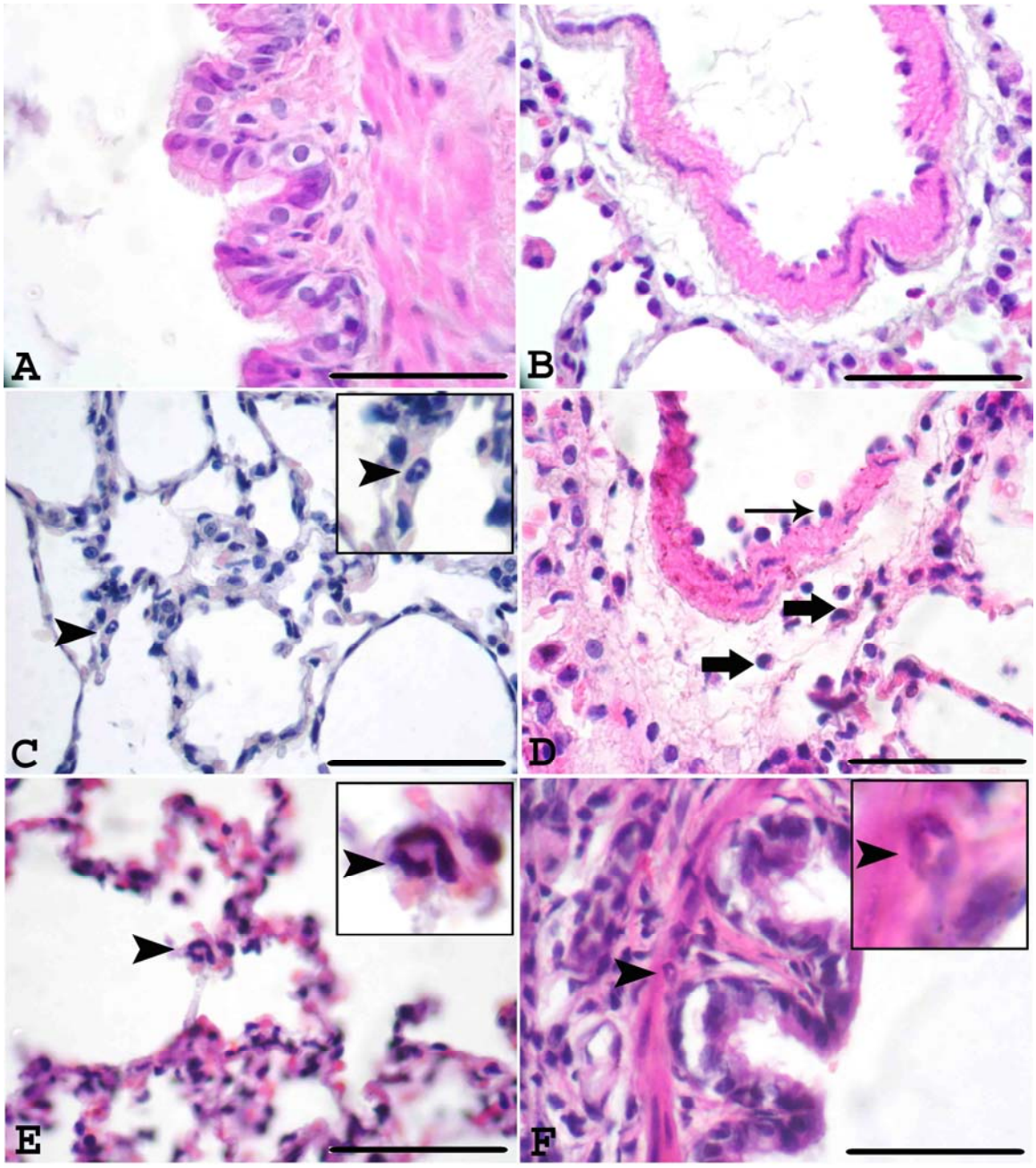

Figure 7.1. Histopathology of lung sections.

Histopathological changes in the lungs of swine barn air exposed, *E. coli* LPS treated and control rats were evaluated using hematoxylin and eosin stained sections. Control rat lung tissues showed no inflammation and normal architecture of the organ (A and B) while rats treated with *E. coli* LPS (C and D) and one day barn exposed rats (E and F) showed septal neutophilic infiltration (arrowhead and inset, C and E), leukocytes adhering to the blood vessel wall (thin arrow, D), perivascular infiltration of leukocytes (thick arrows, D), and peribronchiolar accumulation of neutrophils (arrowhead and inset, F). *Original magnification* A- F: X400 and micrometer bar = 50 µm.

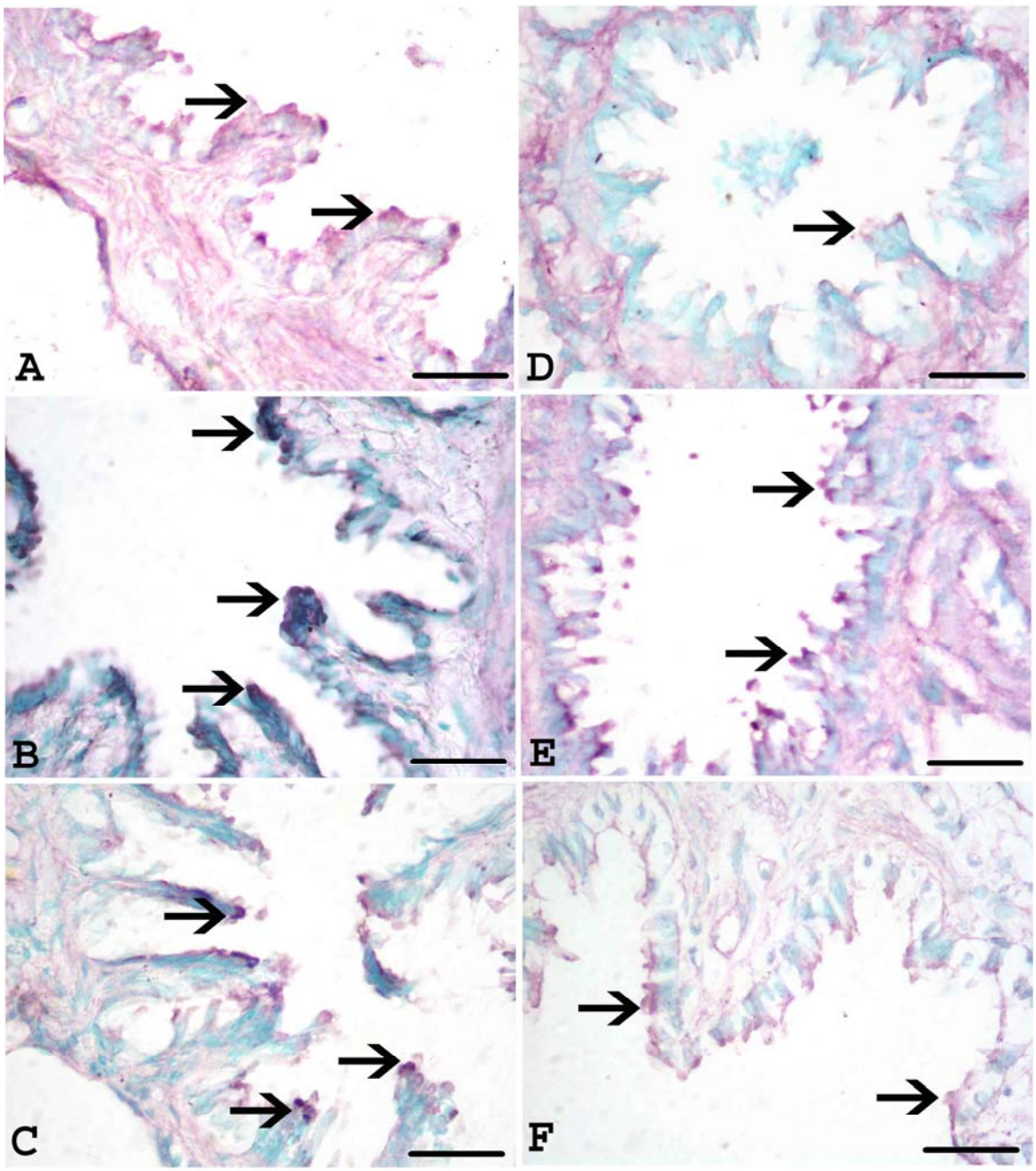

Figure 7.2. Immunohistochemical expression of NMT and CaN in the airway epithelium.

NMT and CaN expression in the lung tissue was detected using immunohistochemistry. Both NMT (A-C) and CaN (D-F) were found to be expressed in the airway epithelium of control (A and D), control + *E. coli* LPS (B and E) and one day barn exposed (C-F) rats (arrows point to the positive cells). Compared to controls, control + *E. coli* LPS and one day barn exposed rat lung sections appeared to have increased expression of both NMT and CaN in their airway epithelium. *Original magnification* A- F: X400 and micrometer bar = 50 μm.

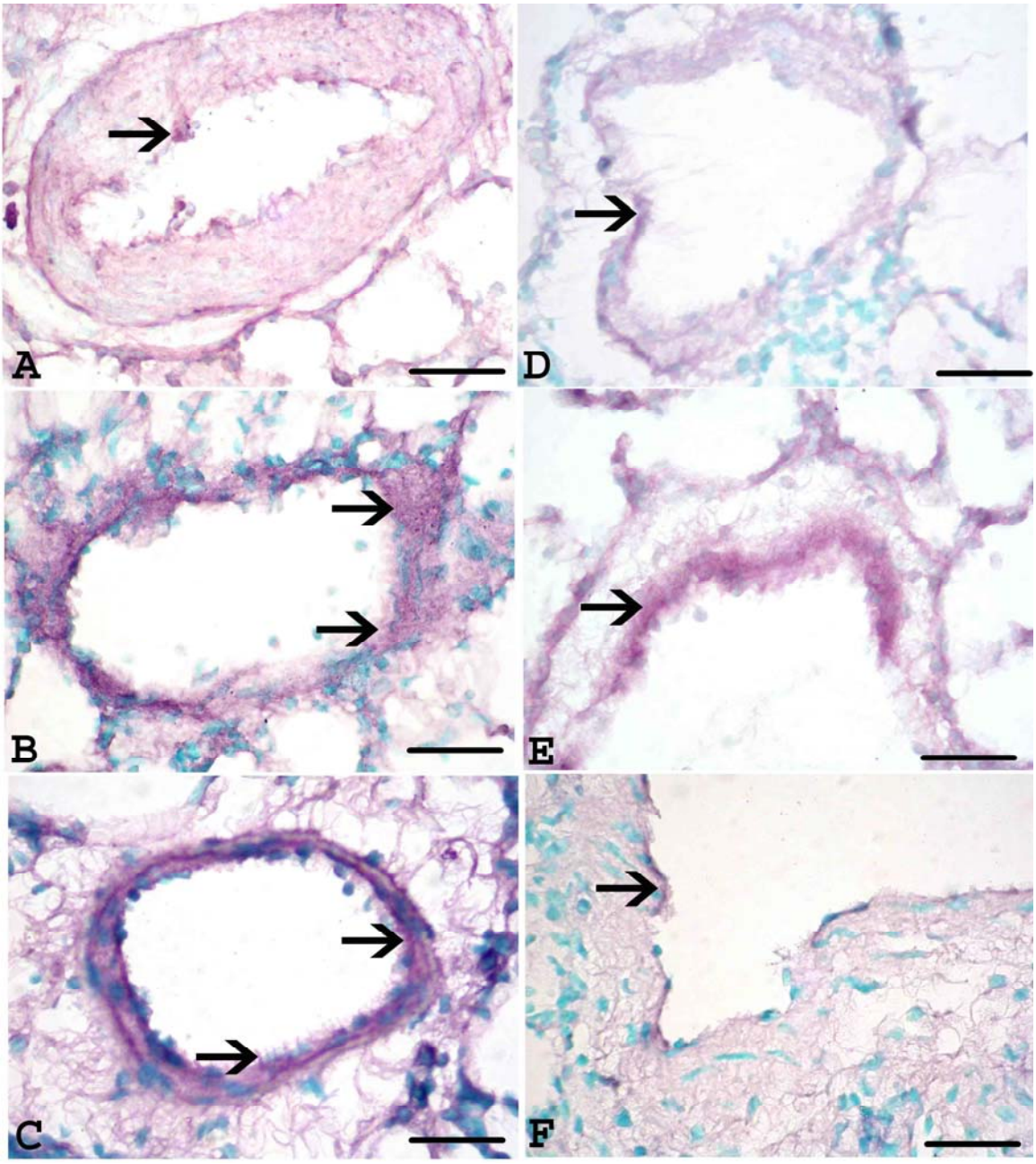

Figure 7.3. Immunohistochemical expression of NMT and CaN in the blood vessels.

NMT and CaN expression in the lung tissue was detected using immunohistochemistry. Both NMT (A-C) and CaN (D-F) were found to be expressed in the blood vessels of control (A and D), control+ *E. coli* LPS (B and E) and one day barn exposed (C-F) (arrows point to the positive cells). Compared to controls, control + *E. coli* LPS and one day exposed rat lung sections appeared to have increased expression of both NMT and CaN in their blood vessels. *Original magnification* A- F: X400 and micrometer bar = 50 µm.

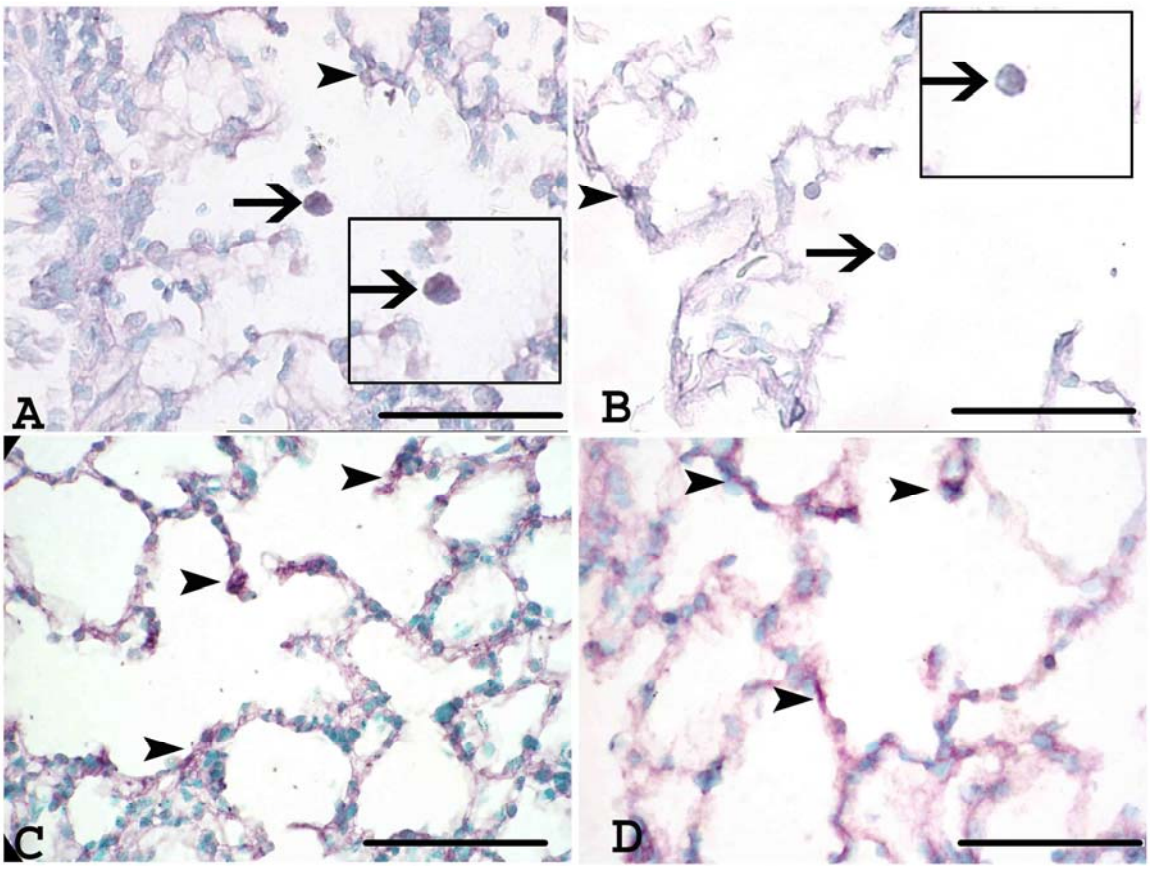

Figure 7.4. Immunohistochemical expression of NMT and CaN in the septa and alveolar macrophages.

NMT and CaN expression in the lung tissue was detected using immunohistochemistry. Both NMT (A and C) and CaN (B and D) were found to be expressed in the septum (arrowheads) and alveolar macrophages (arrows and insets) from control (A and B), control + *E. coli* LPS (C and D) and one day barn exposed (picture not shown) rat lung sections. *Original magnification* A- F: X400 and micrometer bar = 50 μm.

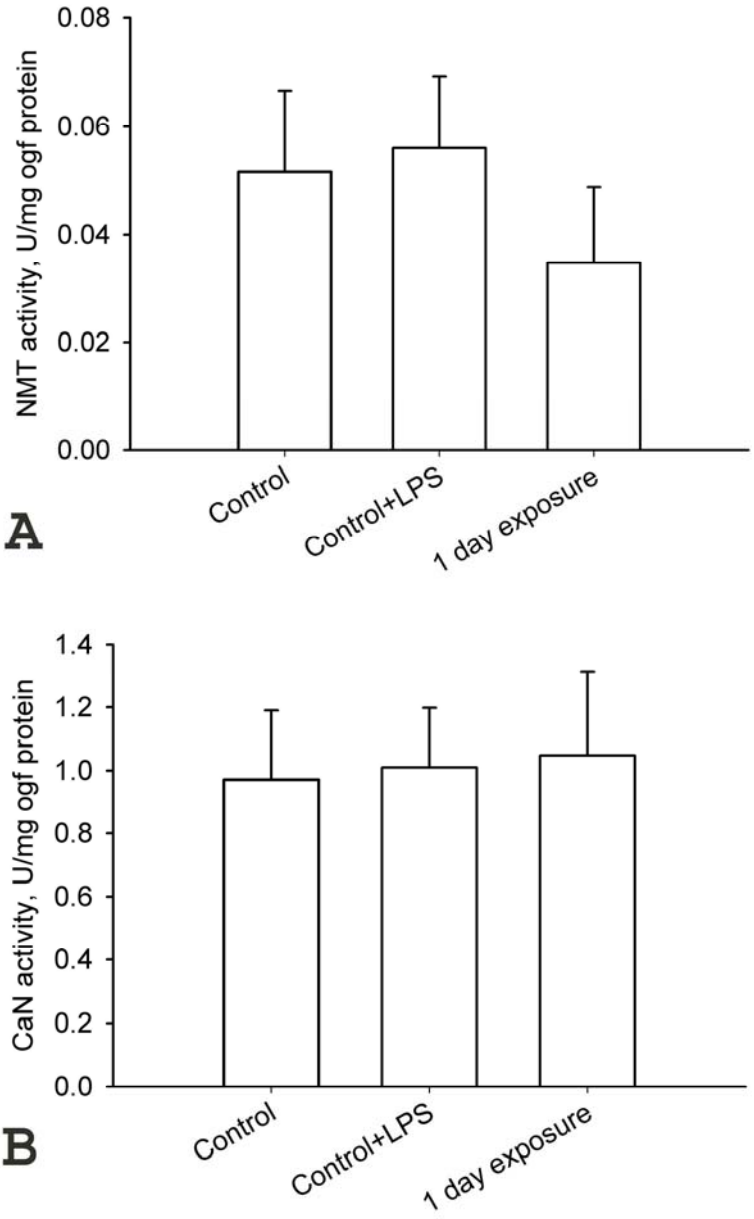

Figure 7.5. Quantification of enzyme activities of NMT and CaN.

NMT (A) and CaN (B) enzyme activities were measured in lung samples. NMT activity was assayed in the tissue extracts using cAMP-dependent protein kinase derived peptide as a substrate while CaN activity was assayed in the presence of 5 mM EGTA and 5 μg CaM/1 mM Ni^{2+} as described under the ‘‘Materials and Methods.’’ The data shown are representative of at least three separate experiments (values are mean ± SD). Both NMT (A) and CaN (B) did not differ in their activities among the three groups ($P>0.05$).

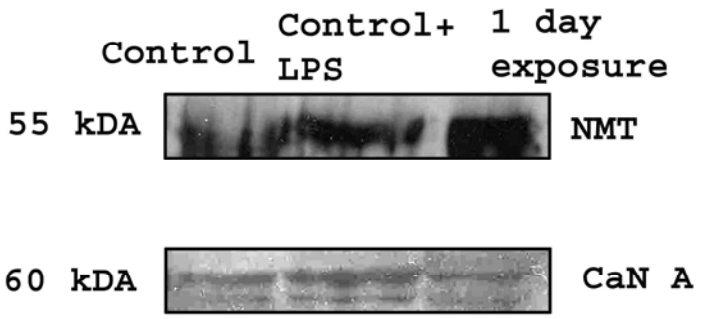

Figure 7.6. Western blotting to detect CaN and NMT proteins.

Western blotting using equal amount of protein from lung extracts (n=3/group) detected both NMT (55 kDa) and CaN (60 kDa) in all the three groups.

7.5. Discussion

I report expression and activities of NMT and CaN in rat models of lung inflammation following either intravenous injection of *E. coli* LPS or a single exposure to swine barn air. The data show that both *E. coli* LPS and single exposure to barn air induce lung inflammation characterized by neutrophilic infiltration, leukocyte margination, perivascular accumulation of leukocytes and peribronchiolar accumulation of neutrophils. Immunhistochemical expression of CaN and NMT was increased in the airway epithelium, blood vessel and lung septae of rats challenged with *E. coli* LPS or exposed to the barn compared to the control. Surprisingly, there was no significant change in the activities of NMT and CaN in rat lung inflammation models.

Recent data has shown the physiological significance of NMT and CaN in myocardial ischemia-reperfusion injury, diabetes, cancer, epilepsy, Alzheimer's disease and inflammation (Sharma, 2004; Selvakumar *et al.*, 2007; Celsi *et al.*, 2007; Lakshmikuttyamma *et al.*, 2008; Rowe *et al.*, 2006; Kang *et al.*, 2007). Now, I have used a well established model of LPS-induced lung inflammation to study expression and activity of both NMT and CaN. Furthermore, I have compared the expression and activities of NMT and CaN in LPS-induced lung inflammation with that initiated by exposure to pig barn air in a rat model, which has been recently characterized as a relevant animal model to study lung dysfunction in pig barn workers (Charavaryamath *et al.*, 2005; Charavaryamath *et al.*, 2008). In the present study, I observed a high expression of NMT and CaN in LPS-induced rat lung inflammation compared to control. Both NMT and CaN proteins were expressed in airway epithelium, alveolar septa, alveolar macrophages and blood vessels. The immunohistochemical expression of NMT and CaN was increased in the airway epithelium, alveolar septal and the vascular endothelium but not in alveolar macrophages following the LPS challenge or exposure to the barn air. I also observed CaN as a single band corresponding to 60 kDa (CaN A). To our knowledge, these are the first data on the expression and activity of two critical signaling proteins in acute lung inflammation induced by two different stimuli.

Both NMT and CaN play important roles in cell signaling (Rusnak and Mertz, 2000; Sieber *et al.*, 2007; Martinez-Martinez and Redondo, 2004; Rowe *et al.*, 2006). One of the fundamental functions of NMT is to facilitate myristoylation of proteins, which are involved in cell signaling (Raju *et al.*, 1995; Johnson *et al.*, 1994; Rajala *et al.*, 2000a; Boutin, 1997; Farazi *et al.*, 2001; Resh, 1999). For example, it is known that myristoylation of TRAM targets it to the plasma membrane, where it is required for LPS responses through the TLR4 pathway while mutated myristoylation site in TRAM resulted in the failure of TRAM to reach plasma membrane and subsequent response to LPS through NF-κB pathway. NMT-induced myristoylation of TRAM, a key adapter protein in the TLR4/NF-κB pathway is involved in innate immunity against LPS (Rowe *et al.*, 2006). CaN regulates the functional activity of LPS-induced NF-κB/Rel proteins in neutrophils (Carballo *et al.*, 1999) as well as IL-8 gene expression through activation of NF-κB pathway (Mitsuyama *et al.*, 2004). Therefore, CaN appears to play critical roles in cell signaling and recruitment of neutrophils largely through NF-κB pathway which is downstream of TLR4 and important for gene transcription of many inflammatory cytokines and chemokines (Doyle and O'Neill, 2006; Boyd *et al.*, 2006). Role of chemokines such as IL-8, which are regulated by CaN, is well established in neutrophil recruitment into inflamed lungs (Mitsuyama *et al.*, 2004). Neutrophilic accumulation is a hallmark of acute lung inflammation observed in lungs from both *E. coli* LPS treated and barn exposed groups. We have recently shown that barn air induced lung inflammation is mainly TLR4 dependent (Charavaryamath *et al.*, 2008) and others have shown involvement of TLR4 in neutrophil recruitment (Takeda *et al.*, 2003; Takeda and Akira, 2005; Andonegui *et al.*, 2003). Therefore, increased expression of NMT and CaN in epithelial and endothelial cells especially in the alveolar septa in lungs may influence cell signaling leading to expression of IL-8, migration of neutrophils and lung inflammation.

I observed similar NMT and CaN activity in both normal and inflamed lungs along with increased immunohistochemical expression in epithelial and endothelial cells in inflamed lungs. Western blotting showed increased NMT protein expression in one day exposed rats while CaN expression was increased in *E. coli* LPS treated rats. Interestingly, the activity of NMT and CaN was not different between inflamed and normal lungs. It is possible that the enzyme activity and expression of proteins may

follow different kinetics due to presence of inhibitor or interacting molecules. Previously, we have demonstrated that the NMT inhibitor protein (NIP71) is homologous to heat shock cognate protein (HSC70) and has NMT inhibitory activity (Selvakumar *et al.*, 2004). Interestingly, increased levels of HSC70 were detected in the brains of normal birds compared to epileptic and carrier fowls (Selvakumar *et al.*, 2005). Because both of the proteins were active in normal and inflamed lungs they would be able to perform their functions. The increase in NMT and CaN expression in alveolar septa, airway epithelium and vascular endothelium in inflamed lungs may be more significant considering their interface with inhaled and vascular stimuli and recruitment of neutrophils. These interesting data require further studies to ascertain precise functions of NMT and CaN in lung inflammation.

The data show that, either intravenous injection of *E. coli* LPS or single exposures to endotoxin rich-swine barn air induce lung inflammation characterized by infiltration of inflammatory cells into the lung. Both *E. coli* LPS and single exposures to barn air increase the expression of NMT and CaN rat lung inflammation models. Understanding the regulation of NMT and CaN by specific inhibitors may help us to control the action of these enzymes on their specific substrates and may lead to improvements in the management of various inflammatory diseases.

CHAPTER 8: GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1. General discussion

The main focus of my research was to investigate the mechanisms of lung inflammation, airway reactivity and loss of lung function following occupational exposure to the swine barn environment. My experiments were aimed at dissecting the roles of inflammatory cells such as macrophages and neutrophils, role of endotoxin and TLR4 signaling, examining the lung responses to a secondary challenge with *E. coli* LPS following exposure to swine barn air as well as other signaling molecules involved in lung inflammation (Figure 1.1).

In my first experiment, I characterized the recruitment and functions of PIMMs in a rat model of *E. coli*-induced sepsis and a secondary challenge with *E. coli* LPS (Chapter 3). Resident PIMMs in few domestic animal species are credited with pro-inflammatory potential while the role of recruited PIMMs in lung inflammation is beginning to emerge (Chang and Ohara, 1994; Singh *et al.*, 1998; Gamage *et al.*, 2007). Therefore, in order to investigate the recruitment and functions of recruited PIMMs, I chose a clinically relevant rat model of *E. coli*-induced sepsis (Singh *et al.*, 1998; Short *et al.*, 1983). My observation of increased numbers of monocytes/macrophages in the lung septae and ultrastructural confirmation of their identity as PIMMs is an early indication of their involvement in sepsis-induced lung injury (Chang and Ohara, 1994; Singh *et al.*, 1998). Further, a secondary *E. coli* LPS challenge during peak recruitment of PIMMs resulted in enhanced lung inflammation, increased levels of TNF-α, IL-10 and TGF-β2 and localization of all these cytokines in recruited PIMMs to indicate a link between recruitment of PIMMs and modulation of lung inflammation.

Findings from my current study indicate a possible mechanism for enhanced lung injury in humans who may develop recruitment of PIMMs under certain circumstances (Dehring and Wismar, 1989; Smith *et al.*, 2004a). Further, transient recruitment of PIMMs following barn exposure and exacerbation of lung inflammation following a secondary *E. coli* LPS challenge in rats (Gamage *et al.*, 2007) raises the possibility of the same risk in naïve barn workers following their first barn exposure. Still there is need for additional experiments to understand molecular mechanisms of recruitment of PIMMs.

In my second study, I modeled full-time barn worker's occupational exposure using a rat model to resolve *in situ* mechanisms of lung inflammation and lung dysfunction (Chapter 4). The data demonstrated that single and five exposures to barn air induce acute lung inflammation as well as increased AHR, while 20 exposures induce chronic lung inflammation with attenuated AHR to indicate adaptive responses. Similar adaptive responses have been seen in swine farmers upon single experimental barn exposure (Larsson *et al.*, 1992; Larsson *et al.*, 1994; Palmberg *et al.*, 2002; Israel-Assayag and Cormier, 2002). Further, the presence of chronic lung inflammation with increased lymphocytes in BALF, mucus cells in the lung and activation of BALT following 20 day exposure suggested persistent lung inflammation and airway remodeling features that may eventually lead to decline in lung function. These data offer partial explanations for annual decline in lung function in swine farmers, while experiencing adaptive airway responses upon experimental exposures. However, it is surprising that acute inflammation and AHR are associated in one and five day exposed rats, while chronic lung inflammation and AHR are discordant in 20 day exposed rats. Interestingly, I recorded high levels of endotoxin in my study setting and suggested a possible link between high levels of endotoxin, acute lung inflammation and increased AHR. However, the cell and molecular mechanisms of lung dysfunction and adaptive airway responses seen in 20 day exposed rats still remain incompletely understood. Further, the specific effects due to endotoxin, peptidoglycan, β-glucans and other harmful agents in the barn need further investigation. Measurement of levels of peptidoglycan, bacterial DNA, β-glucans, ammonia and methane in my study would have been more informative. Nevertheless, this new rat model of occupational exposure to swine barn air recapitulates many features of human health effects of barn exposures and allows many *in*

situ investigations to examine lung inflammation and airway reactivity in the same animal.

In my third study, I addressed the role of endotoxin in the barn air specifically in inducing lung inflammation and AHR by exposing either WT or TLR4 functional mutant mice to barn air, in a fashion similar to my rat model (Chapter 5). Following one, five and 20 day exposures, I showed that lung inflammation but not AHR is dependent on presence of a functional TLR4 to indicate the prime role of endotoxin (Chapter 5). The data demonstrated acute lung inflammation in mice carrying a functional TLR4 following one day exposure while acute inflammatory responses dampened following five and 20 day exposures to indicate prime role of endotoxin in inducing lung inflammation and development of an adaptive response following 20 exposures as seen in my rat model (Chapter 4) and swine farmers (Larsson *et al.*, 1992; Larsson *et al.*, 1994; Palmberg *et al.*, 2002; Israel-Assayag and Cormier, 2002). Surprisingly, increased AHR was seen in both the strains following five day but not one and 20 day exposure to indicate complex regulation of AHR by many injurious molecules in the barn including endotoxin. Further, following 20 exposures, damage to the airway epithelium was seen in both the strains, which I speculate to be due to irritant gases in the barn. My results show a central role of endotoxin in inducing lung inflammation but not AHR. It is interesting that genetic mutations in genes such as *TLR4* and *CD14* have been linked to regulation of innate immune responses to inhaled endotoxin in humans (Arbour *et al.*, 2000; LeVan *et al.*, 2005). My study demonstrates the importance of endotoxin in the barn to induce airway inflammation, which in turn is probably central to various respiratory diseases seen swine farmers. Increased inflammatory cytokines and recruitment of neutrophils seen in WT mice following barn exposure support the endotoxin-induced inflammatory signaling through TLR4. However, my study did not address TLR4-independent regulation of AHR, waning of lung inflammatory responses in five and 20 day exposed mice, decreased AHR and induction of lung epithelial damage in 20 day exposed mice as well as failure of TLR4 mutant mice to develop comparable inflammatory response following exposure to the complex barn environment. Since the nature of the barn environment as well as host innate responses appear complex, future studies examining the roles of other

members of the TLR family and other molecules of the innate immune system will help further our understanding about the barn air induced health effects.

In my fourth experiment, I examined the effect of a secondary challenge with *E. coli* LPS on lung inflammation induced following one or five exposures to the swine barn air (Chapter 6). I demonstrated that one or five exposures to barn air induce lung inflammation and upon secondary challenge with *E. coli* LPS, intensity of lung inflammation increased in one day barn exposed rats treated with *E. coli* LPS with increased granulocyte numbers and IL-1β levels in the lung. However, the increased granulocyte recruitment and IL-1β levels in barn exposed and *E. coli* LPS challenged rats were not different from control rats treated with *E. coli* LPS. Whether the increased lung inflammation in one day barn exposed rats with secondary *E. coli* LPS challenge is due to priming effect of barn exposure or simply due to an additive effect of barn exposure and secondary LPS challenge needs further investigation. However, our results showing increased lung inflammation in one day barn exposed rats following *E. coli* LPS challenge are interesting and may have implications for the health of naïve barn workers who may show an exaggerated response to microbial infections. This is interesting, since long term barn work has shown to induce adaptive responses (Larsson *et al.*, 1992; Larsson *et al.*, 1994; Palmberg *et al.*, 2002; Israel-Assayag and Cormier, 2002). Unlike our previous study (Gamage *et al.*, 2007), I applied a secondary *E. coli* LPS challenge before the peak recruitment of PIMMs and suggested that the observed increase in granulocyte recruitment and IL-1β levels are likely responsible for the exacerbation of lung inflammation. However, my study does not address the specific role of increased recruitment of granulocytes and IL-1β levels observed in robust lung inflammation following secondary *E. coli* LPS challenge. Nevertheless, my study identifies and reconfirms the possible risk for exacerbation of lung inflammation, particularly in newly employed barn workers. The data also show that lungs of animals exposed to barn air remain competent to mount a defense response to secondary challenge.

In my last experiment, I examined the expression and activity of NMT and CaN, both of which have previously been shown to be important in many cell signaling pathways and diseases such as inflammation, cancer, diabetes and myocardial injury (Chapter 7). Hence, I examined the expression and activities of these two key molecules

in lung inflammation induced following single barn exposure or systemic *E. coli* LPS administration. I demonstrated increased immunohistochemical expression of NMT and CaN in lung airway epithelium, blood vessel wall and alveolar macrophages from barn exposed and *E. coli* LPS treated inflamed lungs compared to control lungs. Demonstration of NMT and CaN proteins in endothelial, epithelial and immune cells is critical since both the proteins have shown to play a role in TLR4/NF-κB pathway including chemokinesis, neutrophil, macrophage and lymphocyte functions. Because NMT and CaN are important for myristoylation of proteins and calcium signaling respectively, their increased expression appears to be important in many events of host immune responses. Surprisingly, I did not observe statistical differences in NMT and CaN enzyme activities among the three groups. Based on previous results, I provided a possible explanation for such a discrepancy between protein expression and enzyme activities. Nevertheless, to my knowledge it is the first report of expression of NMT and CaN in rat models of lungs inflammation following either barn exposure or LPS treatment. These data are one of the initial steps towards understating the roles of various other molecules regulating the innate responses to the complex barn environment.

8.2. Conclusions and future directions

To conclude, my experiments have developed credible animal models to study the effects of single and multiple exposures to the barn air, identified the prime role of endotoxin in inducing lung inflammation but not AHR, examined whether there is any priming effect of barn exposure to enhance lung inflammation following a secondary microbial challenge and measured expression and activities of NMT and CaN in barn air induced lung inflammation. My experimental results provide interesting clues to design future logical experiments to expand our knowledge about barn air induced health effects.

In the future, there is a need to measure and characterize the barn air contaminants other than endotoxin and total viable bacteria. Because it is reported that nanoparticles worsen endotoxin induced lung inflammation (Inoue *et al.*, 2006), it will be interesting to see if barn environment contains many varieties of particles in the nano scale. If the barn environment contains a variety of nano-particles, the effect exposure to such particles and interaction of nano-particles with other toxicants in the barn air are worth investigating.

Further, there is a need to design experiments to understand the role of other members of TLR-family in inducing lung inflammation as well as AHR. This is critical since barn environment is complex and contains many microbial and non-microbial injurious agents. For example, barn dust contains bacterial DNA and hence it would be interesting to understand the effect of exposure to bacterial DNA and the role of TLR9 in mediating biological effects of bacterial DNA. Since I have documented the induction of airway epithelial damage (Chapter 5) following 20 day exposure to swine barn air that is independent of TLR4 activation, it is logical to investigate the role of other physical and chemical entities in the barn air that may be involved in causing damage to the lung epithelium. Lastly, there is need to delineate the specific functions of NMT and CaN in barn air induced lung inflammation through functional blocking or other approaches.

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